OBSERVATIONS ON DRUG RESISTANCE IN TUBERCLE BACILLI

by

Sheila M. Stewart, B.Sc.(Lond.)

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Part of the material used for this thesis has been published in the following articles:-

Streptomycin resistance in patients with pulmonary tuberculosis previously treated with P.A.S. alone - Turnbull, F.W.A., Wallace, A.T., Stewart, Sheila and Crofton, J.W. (1953) Brit.Med.J., 1, 1244.

The detection of streptomycin resistance in tubercle bacilli - Stewart, Sheila M. (1955) J.clin. Path., 8, 237.

Studies on the distribution of drug-resistant tubercle bacilli within the lung - Turnbull, F.W.A. and Stewart, Sheila M. (1956) Amer. Rev. Tuberc., 73, 406.

Varied degrees of isoniazid resistance within strains of tubercle bacilli from sputum and pulmonary cavities - Stewart, Sheila M. (1956) Amer. Rev. Tuberc., 73, 390.

Reprints of these papers are filed in the appendix at the end of volume 1.

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The liquid medium tests for P.A.S. resistance and some of the routine resistance tests reported in this thesis were carried out in the City Hospital Laboratory, Edinburgh.

HISTORICAL INTRODUCTION

In 1882 Robert Koch demonstrated that a bacterium, later to be designated Mycobacterium tuberculosis, was the causative organism of tuberculosis. Although this discovery led to the introduction of methods to reduce the dissemination of the disease, it was about 60 years before treatment with specific anti-tuberculous drugs were first used.

Paul Ehrlich (1913), "father of the science of chemotherapy," predicted the use of chemical substances in the treatment of bacterial infections. These compounds, known as chemotherapeutic agents, can be defined as chemical substances which have the capacity to inhibit or destroy micro-organisms in vitro and in vivo at concentrations tolerated by the host. Ehrlich confined most of his chemotherapeutic work to diseases of protozoal or spirochaetal origin, and did little work on bacterial infections. He did however carry out some experiments in vitro with Bechhold (Bechhold and Ehrlich 1906) on the germicidal activity of phenol derivatives.

The first successful treatment of a bacterial infection in vivo with chemotherapeutic agents was reported by Morgenroth and Levy (1911), using ethylhydrocuprein in pneumococcal infections in mice. But the substance proved too toxic for human use. There followed many reports of other animal experiments but no real advance was made in the treatment of human infections until the introduction of the sulphonamide compounds by Domagk in 1935.

The first chemotherapeutic compounds reported with anti-tuberculous /

anti-tuberculous activity were the sulphones (Buttle et al., 1937; Rist et al., 1940; Feldman et al., 1940) and the thiosemicarbazones (Domagk et al., 1946). Neither of these series of substances have been widely used in clinical practice owing to toxicity at effective doses.

In 1946 Lehmann showed that p-amino salicylic acid (P.A.S.) retarded the increased absorption of oxygen by tubercle bacilli brought about by benzoic or salicylic acid (Bernheim 1940). Lehmann also demonstrated and Youmans (1946) later confirmed that P.A.S. possessed bacteriostatic activity for the tubercle bacillus. The successful use of P.A.S. in clinical practice followed shortly afterwards (Vallentin, 1946; Nagley and Logg, 1948; Swedish National Association against Tuberculosis, 1950) and it is still one of the major drugs in use in the treatment of tuberculosis.

A second chemical substance, which is also used extensively, is iso-nicotinic acid hydrazide (isoniazid). It was originally described by Meyer and Mally (1912), but its potentiality as an anti-tuberculous drug was not appreciated until 1951 when its activity in vivo was reported by Fox (1951). Further reports followed (Grunberg and Schnitzer, 1952; Robitzek et al., 1952; Steenken and Wolinsky, 1952; and others).

In the treatment of antibacterial infections a further group of substances has proved of great use to the clinicians - the antibiotics. An antibiotic has been defined by Waksman (1953) as

"a /

"a chemical substance produced by micro-organisms which has the capacity, in dilute solution, to inhibit the growth of, or even to destroy, other micro-organisms". The first antibiotic to be used therapeutically was penicillin. Its activity was first reported by Fleming in 1929 and developed by workers in Oxford (Florey et al., 1946; Florey et al., 1949).

In 1944, Schatz, Bugie and Waksman reported the isolation of an antibiotic substance from Streptomyces griseus which possessed marked anti-bacterial activity in vitro. This substance, known as streptomycin, was found to have relatively low toxicity for man in doses necessary for clinical effectiveness (Hinshaw and Feldman, 1945). It was the first anti-tuberculous drug to be used extensively, and is indeed still one of the major drugs in use in the treatment of this disease.

One of the major disadvantages of the use of chemo-:therapeutic agents and antibiotics has been the emergence of drug-resistant bacilli. This has often proved an unsurmountable obstacle in clinical practice and is particularly evident in the treatment of tuberculosis.

The first reports of streptomycin-resistant organisms were given by Youmans et al. (1946), D'Esopo and Steinhaus (1947), Pyle (1947), Muschenheim et al. (1947), Sadusk and Swift (1948), Wolinsky et al. (1948), Crofton and Mitchison (1948) and the Medical Research Council (1948). In 1949 Sievers, reporting on tests for resistance of /

of tubercle bacilli to P.A.S., commented that at that time no resistant strains had been isolated from patients under treatment with the drug. Later in the same year, however, the first reports of the emergence of P.A.S. - resistant organisms were made. Horne (1949) described a fall in sputum positivity in patients treated with P.A.S., and a subsequent rise after some months of treatment. He suggested that this probably indicated the development of "drug-fast" strains, although no resistance tests were done in his series. Eastlake and Barach (1949), Delaude et al. (1949) and Cartensen (1950) were the first to report the development of P.A.S.-resistant bacilli in patients under treatment, and since then many other workers have confirmed their findings (Nitti et al., 1950 a and b; Daddi et al., 1949, 1950; Veran et al., 1950; Rist et al., 1951 and others). Reports of the emergence of isoniazid-resistant bacilli were made shortly after its use in clinical trials (Bowen and Collins, 1952; Hobby and Lenert, 1952; Knox et al., 1952; Medical Research Council, 1952b; Mitchison, 1952; Petit, 1952).

Ehrlich (1913) in advocating the use of more than one drug suggested that "An advantage of combined therapy is that under the influence of two different medicines the danger of rendering the parasites immune (to arsenic), which naturally would be a very great obstacle in connexion with further treatment, is apparently greatly minimised." The use of combinations of two of the standard drugs, that is, streptomycin, P.A.S. and isoniazid, in the treatment of pulmonary tuberculosis suggested by Demerec (1948) on theoretical grounds /

grounds was shown to be superior, both clinically and bacteriologically, to treatment with any one drug alone (Medical Research Council, 1950, 1952a, 1953a; Tempel et al., 1951; Dye et al., 1953; U.S. Public Health Service, 1953; Pitts et al., 1953; U.S. Veterans Administration, 1953; Mount and Ferebee, 1954). This greater efficiency with combined treatment was however only found when the patients' organisms were sensitive to the drugs in use. If the bacilli were only sensitive to one of the drugs being given, the effect of treatment was similar to that found when that drug was given alone (Turnbull et al., 1953; Medical Research Council, 1953a, 1953c, 1955). It was therefore of great importance to be able to detect <u>in vitro</u> degrees of bacterial resistance which would be of clinical significance in order to help to elucidate failures in treatment.

SCOPE OF THE PRESENT THESIS

The present work is divided into three sections. The first deals with methods for detecting resistance to streptomycin, P.A.S. and isoniazid in tubercle bacilli, as applied to hospital laboratories doing control work on treatment. The methods and variations within the methods are analysed in correlation with details of the patients' previous therapy and sputum examinations, in order to determine the most efficient techniques for the detection of degrees of resistance that are of clinical significance.

The second and third sections deal only with isoniazid-resistant organisms. In section II investigations into the variations in resistance within the sputum are correlated with the case histories in an attempt to determine their significance. Variations within the lung are also reported.

In the third section, some observations are made concerning the virulence of isoniazid-resistant bacilli, the results being correlated with the proportions of the organisms with varying degrees of resistance.

SECTION 1

DETECTION OF DRUG-RESISTANCE IN VITRO

(1) Review of the literature.

(2) Results of the present study.

(a) Normal distribution of drug sensitivity of pre-treatment strains.

(i) Liquid medium (Streptomycin and P.A.S.)

(ii) Solid medium (Streptomycin, P.A.S. and isoniazid)

(b) Strains isolated from patients after treatment

(i) Liquid medium (Streptomycin and P.A.S.)

(ii) Solid medium (Streptomycin, P.A.S. and isoniazid)

(c) Comparison of results by liquid and solid medium on strains isolated after treatment (Streptomycin and P.A.S.)

(d) Tests for the significance of low degrees of resistance (Streptomycin, P.A.S. and isoniazid)

(i) Fall and rise in sputum positivity

(ii) Development of resistance to a second drug

(iii) Persistent sputum positivity

(iv) Clinical and radiological deterioration.

(3) Discussion.

REVIEW OF THE LITERATURE

As long as the treatment of tuberculosis was confined to the use of single drugs, the detection of resistant bacilli in the laboratory was mainly of use as an explanation of clinical failure and also to determine the usefulness of giving a second course of treatment. But with the widespread use of drug combinations, it became of great importance to be able to detect degrees of resistance which were of clinical significance. The large volume of published work concerning methods for the detection of drug resistance in tubercle bacilli and their significance is in itself an indication of the complexity of the subject.

The techniques suggested have varied widely both in the medium used, the inoculum, the time and method of reading the tests and in the definition of "resistant" organisms. The methods are termed "direct" resistance tests when the drug-containing medium is inoculated with the concentrate of the specimen concerned. In "indirect" tests, a primary growth is first obtained on solid medium, and the drug tubes are inoculated from this growth with or without intermediate sub-culture.

The media used may be classified as follows:-

(1) Semi-synthetic liquid medium containing albumin and polyoxyethylene sorbitan monooleate (Tween 80) (Dubos and Davis, 1946).

(2) Coagulated egg-glycerine medium (Löwenstein-Jensen, Mackie and McCartney, 1953; American Trudeau Society, 1952)

(3) Youmans' semi-synthetic medium (Youmans,1946)

(4) /

(4) Egg-agar medium (Herrold, 1931 a and b)

(5) Semi-synthetic medium of Proskauer and Beck (1894)

(6) Slide culture techniques (Pryce, 1941)

In all these methods the drugs are incorporated in the medium. In the case of the coagulated egg media, they are added before inspissation. In some series the slopes of egg-glycerine medium were flooded with the drug solutions after inspissation, before inoculation (Coletsos et al. 1950; Coletsos 1952).

More recently McCabe and Gould (1954) and Knox and Woodruffe (Knox, 1955; Knox and Woodruffe, 1957) have described media which allow a more rapid growth of tubercle bacilli. But no series of pre-treatment or resistant cultures have been reported by these methods so far.

The inoculum used varies considerably from author to author. In tests in liquid media a sub-culture in the medium from the primary growth on egg-glyerine medium is used. Indirect tests on solid medium are made from liquid medium sub-cultures or on suspensions of the growth from the primary culture. Direct tests are carried out on the concentrated specimen.

The results of resistance tests may be given as the minimum concentration of the drug that completely or partially inhibits the growth of the organism. Alternatively the end-point of the titration may be taken as the highest concentration allowing growth equivalent to that in the control tube. In some cases the results /

results are given as a resistance ratio, that is the ratio of the minimum inhibitory concentration for the test organism to the minimum inhibitory concentration for the standard sensitive strain, H37Rv.

A summary is given in tables 1, 2 and 3 of the main series reporting methods for the detection of resistance in tubercle bacilli to streptomycin, P.A.S. and isoniazid respectively. These tables are confined to those reports in which either a series of pre-treatment cultures or definite criteria for resistance have been given.

It will be seen that, where authors are using the same medium, similar results are usually reported for pre-treatment strains. In most of the solid medium techniques, such strains are inhibited by the lowest concentration of the drug tested.

The definitions for "resistance" vary considerably in the different series. For example, strains tested for streptomycin resistance in Dubos and Davis medium may be considered to be resistant if (a) their growth is inhibited by 10 µg. per ml. (Bernstein et al., 1950), (b) if they will grow in medium containing 10 µg. per ml. (Fisher, 1948 a, b; Wolinsky, et al., 1948; Fielding, 1951). or (c) if they are at least four times as resistant as the standard strain (an inhibitory concentration of 2 to 4 µg. per ml.) (Crofton and Mitchison, 1948; Mitchison, 1949).

Although /

Although many authors have indicated that resistance to streptomycin, P.A.S. and isoniazid is of clinical significance, as judged by the effect of treatment on sputum positivity (Crofton and Mitchison, 1948; Horne, 1949; Steenken et al., 1952; Wallace et al., 1954), by the development of resistance on drug combinations (Turnbull et al., 1953; Medical Research Council, 1953 a, 1953c, 1955) and clinical progress (Muschenheim et al., 1947; Rist et al., 1951; Wallace et al., 1954), the criteria for resistance in many of these series was not very clear. There are no published series in which a close correlation has been given between the in vitro resistance tests and details of the case histories.

METHODS

The methods used in the present study to determine resistance of tubercle bacilli to streptomycin, P.A.S. and isoniazid were those most widely used at the time the work was begun, namely serial dilution techniques in Dubos and Davis' liquid medium and in coagulated egg-glycerine medium.

In an attempt to assess the significance of the results obtained by these two techniques, the following investigations were carried out:-

(a) Strains from patients who had never received treatment with the drug were tested in order to determine the normal range of the readings.

(b) Strains from patients who had received treatment with the drug were used to evaluate variations in techniques of the tests and to compare the relative efficiency of the two methods.

(c) The significance of low degrees of resistance was assessed by investigating the effects of treatment on patients whose organisms were of a low degree of resistance.

Media

Tween - albumin liquid medium. The Tween - albumin liquid medium used was that originally described by Dubos and Davis (1946) as modified by the Medical Research Council (1953d). The basic medium had the following formula:-

Potassium /

Potassium dihydrogen phosphate	0·1%
Disodium hydrogen phosphate	0·625%
Sodium citrate	0·15%
Magnesium sulphate	0·06%
Casein hydrolysate (20% casein)	1·0%
"Tween 80"	0·05%

The casein hydrolysate was prepared as follows (Medical Research Council, 1948):-

To 200 g. of commercial casein in a litre conical beaker was added a mixture of 170 ml. of concentrated hydrochloric acid with 110 ml. of distilled water. This was stirred quickly with a glass rod to obtain a uniform suspension before the casein had time to swell and become solid. The mixture was autoclaved at 120°C. for three-quarters of an hour. After cooling, the mixture was made neutral with 40 percent sodium hydroxide and filtered through pulp on a Buchner funnel. The filtrate was diluted to one litre and one percent chloroform was added. It was stored in the dark.

The salts were dissolved one at a time in distilled water and the solution of casein hydrolysate added. The mixture was autoclaved at 10 lb. per sq. in. for 10 minutes. Immediately before use, 4 ml. of a seitz-filtered 9% solution of bovine albumin fraction V was added to 100 ml. medium to give a final concentration of 0·36%.

Dubos medium without Tween. In some tests, the Tween 80 was omitted from the medium.

Löwenstein-Jensen medium. Löwenstein-Jensen egg-glycerine medium was prepared according to the formula given by Mackie and McCartney (1953). A mineral salt solution was prepared with the following constitution:-

Potassium /

Potassium dihydrogen phosphate	0·4%
Magnesium sulphate	0·04%
Magnesium citrate	0·1%
Asparagine	0·6%
Glycerol (A.R.)	2·0%
Distilled water	100 ml.

To 600 ml. of this salt solution were added 30 g. potato starch, and the mixture was heated until it cleared. It was then kept at 56°C for one hour. Hens' eggs, not more than 7 days old, were broken into a flask and the whole shaken mechanically for 15 minutes. The fluid was then filtered through four thicknesses of sterile surgical gauze. One litre of the filtered egg fluid was added to 600 ml. of starch-mineral salt solution. 20 ml. of a 2 percent solution of Malachite Green, which had been incubated at 37°C for 24 hours before use, was added to the 1,600 ml. of egg-starch-salt solution.

Stock solutions of the drugs. Stock solutions of the drugs were prepared in sterile distilled water and stored at 0°C. They were replaced by fresh solutions monthly. The concentrations of these solutions were as follows:-

Streptomycin (as sulphate) 200,000 µg. per ml.
P.A.S. (sodium salt) 10,000 µg. per ml.
Isoniazid 10,000 µg. per ml.

The P.A.S. and isoniazid solutions were not seitz-filtered as no contamination of either medium was encountered.

Liquid medium test.

Preparation /

Preparation of the drug-containing tubes. From the stock solutions two-fold serial dilutions of the drugs in sterile distilled water were prepared at 15 times the final concentrations required in the medium. These were added in a dilution of 1 in 15 to bulk quantities of the medium, and after thorough mixing by hand, the drug-medium solutions were dispensed in 1/4 oz. (7 ml.) screw-capped containers, approximately 3·0 ml. being put into each bottle. The final concentrations of the drugs used were as follows:-

Streptomycin:- 4·0 µg. per ml. by two-fold dilutions to 0·12 µg. per ml. In some tests concentrations of 32 and 128 µg. per ml. were also included.
In the tests carried out in medium without Tween 80, the final concentrations used were 16 µg. per ml. by two-fold dilutions to 0·5 µg. per ml.

P.A.S. (sodium salt):- 2·0 µg. per ml. by two-fold dilutions to 0·03 µg. per ml. Tubes containing 8 and 32 µg. per ml. were also included in some tests. When the medium without Tween 80 was used, the concentrations were 32 µg. per ml. by two-fold dilutions to 0·007 µg. per ml.

Isoniazid tests were not done in Davis and Dubos medium, as this method had already been shown to be inaccurate (Mitchison, 1953; Pansy et al., 1953).

Inoculum:- The primary growth of the organisms on Löwenstein-Jensen medium was sub-cultured into 3·0 ml. Tween-albumin medium and incubated at 37°C for 10 days. Approximately 0·1 ml. of this culture was transferred to a second bottle containing 3·0 ml. of medium. After a further 10 days' incubation, this second sub-culture was used to inoculate the sensitivity sets. The size of the /

the inoculum varied according to the drug being tested. For streptomycin tests, one drop of neat culture (0·02 ml.) was added to each 3·0 ml. quantity of drug-medium mixture. In the P.A.S. tests, it was found (Medical Research Council, 1953d) that if the neat culture was used for the inoculum, sensitive strains grew at high concentrations of the drug. Therefore in these tests an equivalent sized drop (0·02 ml.) of a one-in-ten dilution of the 10-days culture was used.

All tests were incubated at 37°C. Tests for streptomycin resistance were read after 10 and 14 days' incubation. Those for P.A.S. resistance were read after 14 days' incubation only, as the growth was too light at 10 days' due to the small inoculum used. For both drugs, the end-point was taken as the minimum concentration of the drug that inhibited macroscopic growth.

In each batch of tests one culture of the standard H37Rv strain of Mycobacterium tuberculosis (human type) was included. The results were expressed as resistance ratios, that is, the ratio of the minimum inhibitory concentration for the test strain to the minimum inhibitory concentration for the standard strain.

The standard strain was normally inhibited by 0·25 to 0·5 µg. streptomycin per ml. and 0·06 to 0·5 µg. P.A.S. (sodium) per ml. in medium containing Tween 80, and by 1·0 µg. streptomycin per ml. and 0·12 to 0·5 µg. P.A.S. (sodium) per ml. of medium without Tween 80.

Solid /

Solid medium test.

Preparation of drug-containing slopes. Dilutions of the drugs were prepared in sterile distilled water from the sterile stock solutions at concentrations 10 times those required in the medium. The dilutions were added to the medium in bulk quantities in a dilution of one part per 10 parts medium. After mixing by hand, the medium was dispensed in approximately 2 ml. quantities in 1/4 oz. (7 ml.) screw-capped bottles. The drug-medium mixture was then inspissated in the sloped position at 75 - 80°C for one hour. The actual concentrations of the drugs in the medium before inspissation were as follows:-

Streptomycin:- 64 µg. per ml. by two-fold dilutions to 1 µg. per ml.

P.A.S. (sodium salt):- 32 µg. per ml. by two-fold dilutions to 0·25 µg. per ml.

Isoniazid:- 0·2, 1·0, 5, 10 and 50 µg. per ml. In some tests a concentration of 0·06 µg. per ml. was also included.

With each set of dilutions of each drug, a slope was included which was prepared with sterile distilled water instead of drug dilution. This tube acted as a control on the growth of the inoculum.

Inoculum:- A suspension of the organisms was prepared by shaking a loop-ful of a 3-6 weeks primary growth on a Löwenstein-Jensen slope in 0·3 ml. sterile distilled water with glass beads in a mechanical shaker for 30 minutes. A 3 mm. loopful of the resulting /

resulting suspension was streaked up the centre of the slope, care being taken not to dip the loop into the water of condensation at the foot of the slope. The same inoculum was used for all drugs.

All tests were incubated for four weeks at 37°C. The end-points for the streptomycin and P.A.S. tests were taken as the lowest concentration totally inhibiting the growth ("no growth" end-point), inhibiting the growth of 20 or more colonies ("20-colony" end-point) and inhibiting the growth of 100 or more colonies ("100-colony" end-point). The end-points for the isoniazid resistance tests on pre-treatment cultures were the same as those used for the streptomycin and P.A.S. tests given above. For all other cultures, the results were expressed as the concentration allowing any degree of growth (the "no growth" end-point), allowing the growth of 20 or more colonies (the "20-colony" end-point) and allowing the growth of 100 or more colonies (the "100-colony" end-point).

One culture of the standard sensitive strain, H37Rv, was included in each batch of tests. The results of the streptomycin and P.A.S. tests were expressed as resistance ratios to the standard H37Rv strain. Those for isoniazid were given as the minimum concentration inhibiting growth (within the definitions given above) for pre-treatment strains, and as the highest concentration allowing the growth of other strains.

The standard strain (H37Rv) was normally inhibited by 1 to 4 µg. streptomycin per ml., and 0·5 to 2 µg. P.A.S. (sodium salt) per ml. The standard strain was usually inhibited by 0·2 µg. isoniazid per ml. but irregularily by 0·06 µg. per ml.

RESULTS

NORMAL DISTRIBUTION OF RESISTANCE OF PRE-TREATMENT STRAINS

Streptomycin.

Liquid medium with Tween 80. Forty-nine pre-treatment cultures from 49 patients, that is, 49 strains, were tested for streptomycin resistance in liquid medium with Tween 80. The detailed results after 10 and 14 days' incubation, expressed as minimum inhibitory concentrations in µg. streptomycin per ml. and as resistance ratios to the standard H 37Rv strain are given in table 4, and are summarised in table 5. In this series, the standard strain was inhibited by 0·25 to 0·5 µg. per ml. at 10 days and by 0·25 to 1·0 µg. per ml. at 14 days.

There was no significant difference between the 10 days and 14 days readings. The majority of the strains were inhibited by 0·25 µg. per ml. and were at least as sensitive as the standard strain. In 19 of the 49 strains, no growth occurred at the lowest concentration tested after 14 days' incubation and consequently no absolute readings could be obtained. It was therefore not possible to submit these results to statistical analysis. All the strains tested were inhibited by 0·5 µg. per ml. or less and were not more than twice as resistant as the standard strain. Therefore a minimum inhibitory concentration of 1·0 µg. per ml. or over or a resistance ratio of four or above would indicate an increase in resistance /

resistance outwith the normal range of pre-treatment sensitive cultures for both 10 days and 14 days readings.

Solid medium. Table 6 shows the results of solid medium resistance tests to streptomycin on 51 pre-treatment strains. These are summarised in table 7. The results, after 2 and 4 weeks' incubation, are expressed as minimum inhibitory concentrations and as resistance ratios, taking as the end-point of the titrations total inhibition of growth (no growth end-point), inhibition of 20 or more colonies (20-colony end-point) and inhibition of 100 or more colonies (100-colony end-point). It will be seen that no growth occurred even on the control tubes at the end of 2 weeks' incubation in 21 of the 51 strains tested. This incubation period would therefore seem to be too short to allow of growth of many strains after primary isolation. All of the remaining 30 cultures, from which a reading was possible, required less than 16 µg. per ml. for inhibition of growth by any end-point and none was more than four times as resistant as the standard strain.

After 4 weeks' incubation all but one of the 51 strains were inhibited by 8·0 µg. per ml. or less regardless of the end-point taken. This single strain was inhibited by 16 µg. per ml. The majority of the cultures were not more than twice as resistant as the standard strain. A resistance ratio of four was however obtained from two cultures with the "no growth" or "20-colony" end-point and from four with the 100-colony end-point.

The /

The results of the analysis of variance for the minimum inhibitory concentrations and for the resistance ratios for the three end-points after 4 weeks' incubation is given in table 8. The cultures in which an absolute reading was not possible (i.e. with m.i.c. of less than 1·0 µg. per ml. or a resistance ratio of less than ½) have not been counted in the analysis. Therefore the final results may be slightly higher than if a full titration had been done on these strains. The table shows that, at the 99 percent level, the lowest reading outside the normal range is a minimum inhibitory concentration of 16·0 µg. per ml. for all end-points. When expressed as a resistance ratio, the lowest figure is 4 for the "no growth" end-point for P=0·01 or 0·02. When the "20-colony" and "100-colony" end-points are considered, the readings are 8 and 4 for P=0·01 and 0·02 respectively.

P.A.S.

Liquid medium with Tween 80. Fifty-two pre-treatment strains were tested for P.A.S. resistance in liquid medium with Tween 80. The results after 14 days' incubation, expressed as the minimum inhibitory concentrations in µg. P.A.S. (sodium) per ml. and as resistance ratios, are given in table 9 and summarised in table 10. As a one in ten dilution of the inoculum was used for these tests, the growth after 10 days incubation was too weak to allow of a reading at that time. The standard strain was inhibited by 0·06 to 0·5 µg. sodium P.A.S. per ml. in this series.

All /

All but two of the 52 cultures tested were inhibited by 0·25 µg. per ml. or less. In the remaining two strains the minimum inhibitory concentration was 0·5 µg. per ml. Two strains were four times as resistant as the standard strain. The remaining 50 cultures were not more than twice as resistant as the H 37Rv strain.

When the analysis of variants is applied to these results, the variance is found to be 0·9212 for the minimum inhibitory concentration readings and 1·1026 for the resistance ratios. The corresponding figures for the standard deviations are 0·9160 and 1·009 respectively. The lowest readings outside the normal range at the 99 percent level are a m.i.c. of 0·5 µg. per ml. or a resistance ratio of 8. At the 98 percent level a ratio of 4 is just significant.

Solid medium. Table 11 shows the results of solid medium resistance tests to P.A.S. on 52 pre-treatment cultures. The results, for the "no growth", "20-colony" and "100-colony" end-points, are expressed as minimum inhibitory concentrations of P.A.S. (sodium) per ml. and as resistance ratios. These are summarised in table 12.

In twelve of the cultures less than 100 colonies grew after 2 weeks' incubation. Of the remaining 40 strains, all but one were inhibited by not more than 2·0 µg. sodium P.A.S. per ml. and all were less than eight times as resistant as the standard strain. There was no significant difference between the readings using the three different end-points.

After /

After 4 weeks' incubation, one strain required 8·0 µg. per ml. for inhibition of any degree. The remaining 51 cultures were inhibited by 4·0 µg. per ml. or less. No strain was more than four times as resistant as the standard strain except for one which was 8 times as resistant at the 100-colony end-point.

Table 13 shows the analysis of variance for the 4 weeks' readings. Where the tirtration was too low for an end-point to be found, the results have been ignored in the statistical analysis. At the 99 percent level, a minimum inhibitory concentration of 16·0 µg. per ml. at the "no growth" end-point, or 8·0 µg. per ml. at the "20-colony" or "100-colony" end-points, and a resistance ratio of 8 by all end-points were found to be the lowest readings outside the normal range.

Isoniazid.

Tests for isoniazid resistance were only made on solid medium. The readings on 102 pre-treatment strains after two and four weeks' incubation, given in table 14, are summarised in table 15. The results are expressed as the minimum inhibitory concentrations of isoniazid in µg. per ml. completely inhibiting growth ("no growth" end-point), inhibiting the growth of 20 or more colonies ("20-colony" end-point) and that inhibiting the growth of 100 or more colonies ("100-colony" end-point).

In eleven of the strains, the growth consisted of less than 100 colonies after two weeks' incubation. Using the "no growth" end-point, /

end-point, one culture required 1·0 µg. per ml. for inhibition and one 5·0 µg. per ml. The remaining strains were all inhibited by 0·2 µg. per ml. After 4 weeks' incubation, taking the 20 and 100 colony end-points, all of the 102 strains were inhibited by 0·2 µg. per ml. When the "no growth" end-point was used, 6 strains required 1·0 µg. per ml. for inhibition, two 5·0 µg. per ml. and one 10·0 µg. per ml.

50 pre-treatment strains were tested for resistance to 0·06 µg. per ml. in addition to the normal range of concentrations. In 12 of these strains, a growth of 20 or more colonies occurred on the slope containing 0·06 µg. per ml. after 4 weeks' incubation.

These results suggest that by this technique a growth of 20 or more colonies on the slope containing 0·2 µg. isoniazid per ml. or more indicates a strain of greater resistance than that found in the normal pre-treatment range. Growth at 0·06 µg. per ml. occurred too frequently in the normal sensitive population for this concentration to be of use in detecting resistance.

Comparison of different series of pre-treatment strains examined in two separate laboratories.

In order to ascertain whether there was any variation in the normal pre-treatment readings found in different laboratories using the same techniques, the results of the present series were compared with series reported from the Postgraduate Medical School (by courtesy of Dr. D. A. Mitchison). These comparisons are confined /

confined to streptomycin and P.A.S. resistance tests in liquid medium with Tween 80 read at 14 days and in solid medium read at 4 weeks using the 20-colony end-point. The results are given in table 16.

The range of minimum inhibitory concentrations varied between the laboratories for the same drug and medium. In contrast, the range of resistance ratios for each drug in a given medium was very similar. It seems therefore, that in order to obtain comparable figures between laboratories it is necessary to express the results as resistance ratios to a standard sensitive strain rather than as absolute concentrations of the drug.

For this reason, all the results for streptomycin and P.A.S. resistance tests in this thesis are expressed as resistance ratios to the standard H37Rv strain. The isoniazid tests, carried out by the method recommended by the Medical Research Council, are given as the concentration of the drug allowing the growth of 20 or more colonies. This method has proved satisfactory in clinical use (see later in this thesis) and has not therefore been altered.

Summary.

Table 17 gives a summary of the readings found to be outside the normal range of pre-treatment strains for the various methods considered above. Although accuracy at the 99 percent level would be desirable for the streptomycin and P.A.S. tests, in practice the 98 percent level is more useful. By this reading two strains per hundred /

hundred would be classified as resistant which were actually sensitive to the drug. If however two or more cultures were tested from a patient, the probability of both showing falsely as resistant would be only one in 250 cases. Therefore the readings for P = 0·02 have been used in comparing the efficiency of the various methods to detect resistant strains.

STRAINS ISOLATED FROM PATIENTS AFTER TREATMENT.

In order to determine which of the above methods and criteria were most satisfactory for detecting resistance in tubercle bacilli, readings obtained with strains isolated from patients after treatment with the drugs were compared. Where discrepancies occurred, the details of these cases were analysed to decide which method gave the truest indication of the clinical response to the drug.

Streptomycin.

Liquid medium. The 10 days' and 14 days' readings of streptomycin resistance tests in liquid Dubos medium with Tween on 100 strains isolated from patients who had received the drug are given in table 18 and summarised in table 19. Fifty-eight cultures were sensitive and 37 were resistant at both the 10 days' and 14 days' readings. The remaining five strains were all sensitive by the 10 days' reading but resistant by the 14 days' reading. In three of these five cases (SR 13, SR 46 and SR 74) at least three other cultures isolated from each patient had given resistance ratios of 8 or more to streptomycin. In the remaining two cases, (SR 50 and SR 53), resistance was suspected, as the sputum had remained persistantly positive on treatment with intermittent streptomycin and daily P.A.S., although resistance had not been demonstrated *in vitro*.

On these results, the 14 days' readings seemed preferable

to /

to the 10 days' reading for detecting streptomycin resistance in liquid medium with Tween 80.

Solid medium. The results of streptomycin resistance tests on solid medium on 100 strains isolated from patients who had received treatment with the drug are given in table 20. The readings, using the "no growth", "20-colony" and "100-colony" end-points are summarised in table 21. Of the 40 cultures sensitive by the 20-colony end-point, 39 were also sensitive by the no-growth and by the 100-colony end-points. The discrepant strain (SR142) was from a patient whose organisms had become isoniazid resistant during treatment with daily streptomycin and isoniazid, and in whom a resistance ratio of greater than eight for streptomycin had been found on four previous occasions.

The remaining 60 cultures were all resistant by the 20-colony end-point. Of these, all but two (SR113 and SR 115) were also resistant by the "no growth" end-point. One of these two cultures (SR 113) had been isolated from a patient whose bacilli had developed resistance to isoniazid on daily streptomycin and isoniazid, and from whom cultures with resistance ratios of greater than eight by the liquid medium had been isolated previously. The second culture (SR 115) was not inhibited by 64·0 µg. streptomycin per ml., but owing to a growth of less than 20 colonies of the standard strain in the 64 µg. per ml. tube, the ratio was "greater than 1". Three other cultures from this patient had given resistance ratios of 4 on the /

the solid medium test. It was therefore probable that in fact the bacilli were resistant to the drug.

Only one of the 60 strains resistant by the 20-colony end-point appeared sensitive by the 100-colony end-point (SR 178). In this patient isoniazid resistance had developed on treatment with daily streptomycin and isoniazid.

To summarise, there were three strains which gave a resistance ratio of four or over with the 20-colony end-point, but which were sensitive by either the "no growth" or 100-colony end-points. In all these cases there was reason from the previous chemotherapeutic history of the patients and from previous resistance tests to expect the strains to be streptomycin resistant. On the other hand, only one strain, which on analysis was expected to be resistant, was demonstrated to be so by the "no growth" and 100-colony end-points but not by the 20-colony end-point.

The differences between the readings by the various end-points were slight, but they suggested that the 20-colony end-point was slightly more sensitive than the other two in detecting resistant organisms. Therefore this end-point only has been used for the remainder of the work reported here. A resistance ratio of four or above on two or more cultures from the same patient has been taken as indicative of resistant organisms.

P.A.S.

Liquid /

Liquid medium. Owing to the low inoculum used for the P.A.S. resistance tests in liquid medium, and the consequent slow appearance of growth, no 10 days' readings were possible. Therefore the readings after 14 days' incubation was used. A resistance ratio of four has been taken as indicating resistant organisms, since this was shown to be outwith the normal range at the 98 percent level.

Solid medium. Details of the readings of P.A.S. resistance tests on 100 cultures isolated from patients who had received treatment with the drug are given in table 22. The results are summarised in table 23. All of the 52 cultures which were sensitive by the 20-colony end-point were also sensitive by the "no growth" end-point. Three of these 52 cultures were apparently resistant by the 100-colony end-point; one strain (PR 71) was from a patient who had been treated with P.A.S. alone for nine months, and from whom three previous cultures had been isolated which gave a resistance ratio of greater than 8 on solid medium. The second strain (PR 64) was isolated from a patient whose organisms had become resistant to streptomycin on treatment with intermittent streptomycin and P.A.S. followed by daily streptomycin and P.A.S., but in whom resistant organisms had not been demonstrated in vitro. The bacilli were therefore considered to be of "doubtful" resistance. The third strain (PR 5) was from a patient in whom sputum conversion had occurred with treatment with daily streptomycin and P.A.S. followed by /

by intermittent streptomycin and P.A.S. The bacilli were therefore probably sensitive in this case.

Of the 48 cultures which were resistant by the 20-colony end-point, one was sensitive by the "no growth" end-point (PR1) and two by the 100-colony end-point (PR 56 and PR 98). Strains PR 1 and PR 98 were in fact known to have given resistance ratios of greater than 8 on previous resistance tests. The third strain (PR 56) was shown to be sensitive on repeat testing.

The differences between the readings are not therefore very marked. There is a slight bias towards the 20-colony end-point. This end-point has therefore been used, strains being considered resistant if a ratio of eight or over is obtained.

Isoniazid.

Solid medium. Table 24 gives the readings, for the three end-points, of isoniazid resistance tests in solid medium on 50 strains isolated from patients who had been treated with the drug. A comparison of the results is given in table 25. One strain (HR 5) appeared sensitive on the 100-colony end-point but resistant by the 20-colony end-point. Other cultures from this patient had repeatedly shown growth on medium containing 50 µg. isoniazid per ml., and subsequent treatment with daily streptomycin, P.A.S. and isoniazid, when her bacilli were also resistant to the former two drugs, failed to convert her sputum.

Owing /

Owing to the wide scatter of the readings of pre-treatment strains using the "no growth" end-point (see table 15), a reading of 10 µg. per ml. is necessary to indicate resistance. By this criterion, 26 of the 48 strains resistant by the 20-colony end-point would have been classified as sensitive. The case histories and other resistance tests had shown all of these 48 strains to be resistant.

Although there was no difference between the 20-colony and the 100-colony end-point results, the 20-colony end-point has been chosen for routine use in order to have a uniformity in the readings for streptomycin, P.A.S. and isoniazid tests. The wide variations of pre-treatment cultures with the "no growth" end-point made this reading of little practical use. Strains have been considered resistant to isoniazid if a growth of 20 or more colonies was obtained on the slope containing 0·2 µg. per ml.

Summary.

To summarise, the 14 days reading in liquid medium streptomycin tests and the 20-colony end-point readings for all solid medium tests after 28 days' incubation were found to give the closest correlation with the chemotherapeutic histories and with the clinical response of the patients.

COMPARISON OF RESULTS OF RESISTANCE TESTS ON SOLID AND IN LIQUID MEDIA

In order to determine which of the methods discussed above was the most satisfactory for use in detecting the presence of streptomycin and P.A.S. resistant bacilli, tests were carried out in liquid medium and in solid medium on the same cultures. As a discrepancy was demonstrated between the results of tests on these two media, some strains were also tested in liquid medium without Tween 80. The results in this medium were compared with those in liquid medium with Tween and with those on solid medium.

Streptomycin.

The detailed results of streptomycin resistance tests carried out in liquid medium with and without Tween 80 and in solid medium are given in table 26.

Liquid medium with Tween 80 and solid medium. A comparison of the readings of resistance tests on solid medium and in liquid medium with Tween on 53 strains from patients who had received the drug is given in table 27. A resistance ratio of 4 is taken to indicate resistance by either method. 17 of the strains were resistant and 19 sensitive in both media. Of the remaining 17 cultures, 15 were resistant when tested in solid medium, but sensitive in liquid medium with Tween. All but 4 of these 15 strains had a resistance ratio of 8 or over on solid medium. Only two strains were resistant on /

on liquid medium, but sensitive on solid medium. An analysis of the strains showing discrepancies between the two media is given in table 28. It will be seen that 13 of the 15 cultures resistant on solid medium only were in fact expected to be resistant by virtue of the previous treatment of the patient from whom the strain was isolated or from previous resistance tests on other cultures. In two, the presence of resistant organisms was classed as "doubtful" although the previous treatment was such that resistant organisms were very likely to have developed. Of the two strains that appeared sensitive on solid medium but resistant by liquid medium, one was in fact resistant, the organisms having become isoniazid resistant on treatment with daily streptomycin and isoniazid. Subsequent cultures were also shown to be resistant on solid medium. The other strain was likely to be sensitive to the drug, as the patient had not received any treatment before the culture was isolated from the sputum, and subsequent treatment with daily streptomycin and isoniazid was completely satisfactory. Two other cultures tested from this patient on liquid medium were fully sensitive to the drug.

These results suggest therefore, that the solid medium test is more efficient than the test in liquid medium with Tween for detecting streptomycin resistant organisms.

Liquid medium without Tween and solid medium. A summary of the readings of tests on solid medium and in liquid medium without Tween are given in table 29. A resistance ratio of 4 was taken as indicative /

indicative of the presence of resistant organisms in either medium. Of the 31 cultures tested, 10 were sensitive and 17 resistant by both methods. An analysis of the remaining 4 cultures is given in table 30. It will be seen that in three cases, (SR 224, SR 228, SR 230), the bacilli were expected to be resistant by previous chemotherapy or because of the results of previous resistance tests. In the remaining case (case SR201) resistance had not previously been demonstrated, but the previous treatment of the patient was such that resistant organisms were very likely to have developed.

Therefore the solid medium test seemed slightly preferable to the liquid medium without Tween, although the differences were not very marked.

Liquid medium with and without Tween. The results of tests in liquid medium with and without Tween are summarised in table 31. A ratio of 4 is taken as indicative of resistance in both media. The results in the two media were in agreement in 26 cases, 14 being sensitive and 12 resistant. All of the remaining 5 cultures were resistant in medium without Tween but sensitive in medium with Tween. An analysis of these five cultures is given in table 32. All were expected to be resistant.

Therefore, although the numbers are small, results of these comparisons suggest that medium without Tween is more satisfactory than that containing Tween, but that neither medium is as efficient as solid medium.

P.A.S. /

P.A.S.

The results of P.A.S. resistance tests on solid medium and in liquid medium with and without Tween 80 on 71 cultures isolated from patients who had been treated with the drug are given in detail in table 33.

Liquid medium with Tween and solid medium. The results of tests carried out in solid medium and in liquid medium with Tween 80 are summarised in table 34. A resistance ratio of 4 is taken as indicative of resistance in the liquid medium with Tween and of 8 in the solid medium. Of the 71 strains tested, 27 were resistant and 25 sensitive by both methods. Of the remaining 19 cultures, 17 were resistant on solid medium only, and two were resistant only in liquid medium. An analysis of these 19 cultures is given in table 35.

Of the 17 cultures resistant on solid medium only, 15 were expected to be resistant. The other two were of doubtful resistance, details of therapy and previous tests not being available. Of the two cultures resistant on liquid medium only, one was likely to be resistant, since the bacilli became resistant to streptomycin when the patient was treated with daily streptomycin and P.A.S. The other was of doubtful sensitivity.

These results suggest that the solid medium is superior to the liquid medium with Tween 80 in detecting P.A.S. resistance.

Liquid /

Liquid medium without Tween and solid medium. The results of tests on strains in liquid medium without Tween 80 and on solid medium are compared in table 36. A ratio of 4 in liquid medium without Tween 80 and of 8 in solid medium has been taken as indicating resistance. In 43 of the 57 cultures tested, the results by the two methods were in agreement, 13 being sensitive and 30 resistant. All of the remaining 14 cultures were resistant in liquid medium but sensitive in solid medium. A detailed analysis of these strains is given in table 37. Five were expected to be resistant, three were "doubtfully" resistant and six were sensitive. Therefore, in both media, some strains failed to show a correlation with the clinical details of the case.

Liquid medium with and without Tween. Table 38 gives a summary of the results of tests for P.A.S. resistance in liquid medium with and without Tween 80. A resistance ratio of 4 is taken to indicate the presence of resistance in either medium. 13 cultures were sensitive and 18 resistant by both methods. All of the remaining 26 strains were sensitive in medium containing Tween 80 but resistant in medium without Tween. An analysis of these cultures is given in table 39. 17 of the strains were expected to be resistant, two were of "doubtful" resistance and six were sensitive. In one case, details were not available.

Therefore, although the liquid medium without Tween did in some cases indicate resistance in an apparently sensitive strain, that /

that medium seemed preferable to the medium with Tween for detecting P.A.S. - resistant strains.

Summary.

The results reported here show that Löwenstein-Jensen medium is superior to Dubos medium with or without Tween 80 for detecting strains of tubercle bacilli resistant to streptomycin or P.A.S. A comparison of the solid medium and liquid medium tests with those carried out in liquid medium without Tween show that the tests in the absence of Tween agree more closely with the solid medium ones than do those with Tween. This suggests that Tween 80 is partly responsible for the suppression of resistance in liquid medium tests.

SIGNIFICANCE OF LOW DEGREES OF RESISTANCE.

Many of the methods advocated for the routine testing of drug resistance ignore the lower levels of resistance. These have nevertheless been found by present methods to be outwith the normal range of sensitivity of pre-treatment strains. The levels concerned are resistance ratios of 4 and 8 to streptomycin and 8 and 16 to P.A.S. and a maximum concentration of 0·2 and 1.0 µg. isoniazid per ml. allowing the growth of 20 or more colonies. In order to assess the significance of these low levels, 136 cases whose organisms were known to be resistant to one or more drug were investigated. The cases were mainly those which had been included in research trials of the Medical Research Council or were later admitted to local trials of viomycin or pyrazinamide. There was therefore no selection of cases. Full bacteriological details were not available on all cases. Where there was sufficient information, the following criteria were used to indicate that a patient's organisms were failing to be affected by the drug and that the degree of resistance detected was therefore of clinical significance:-

1) Initial fall in sputum positivity after the start of treatment followed by a rise at the time of emergence of resistance to the drug.

2) The development of resistance to a second drug during combined therapy, when the organisms were known to be resistant to the /

the first drug at the start of treatment.

3) Persistent sputum positivity of at least six months' duration from the start of treatment, when the bacilli were known to be resistant to the drugs being given by the end of treatment.

4) Clinical or radiological deterioration while under treatment with the drug or drugs in question.

In the 136 cases investigated, the bacilli were resistant to streptomycin in 111, to P.A.S. in 105 and to isoniazid in 101. Of these, the percentage with organisms of a low degree of resistance was 30, 34 and 26 respectively. These figures can be taken as representing the incidence of organisms of low degrees of resistance in an average resistant population, since the 136 cases were not selected in any way.

If organisms of a low degree were not of clinical significance, it would be expected that the incidence of patients excreting only bacilli of low degrees of resistance in cases which presented evidence of clinical significance of resistance as judged by the above criteria would be markedly less than in the average resistant population. Therefore the incidence of cases with organisms of a low degree of resistance showing signs of significance of resistance has been compared with the incidence of such cases in the average resistant population.

Fall and rise of sputum positivity.

A summary of the results of resistance tests on cultures isolated /

isolated before and after treatment together with the sputum positivity before and during treatment, for cases resistant to streptomycin, P.A.S. and isoniazid are given in tables 40, 41 and 42 respectively. Detailed examples for each drug are given in figures 1, 2 and 3.

The incidence of low degrees of resistance to streptomycin, P.A.S. and isoniazid in cases in which a fall and rise in sputum positivity was demonstrated, is compared in table 43 with the percentage of cultures of low degrees of resistance in the average resistant population. It will be seen that the incidence of bacilli of low degrees of resistance was similar in the cases in which a fall and rise was demonstrated to that in the average resistant population.

Development of resistance to a second drug.

In table 44 are given the details of resistance tests and treatment for 18 patients whose organisms developed resistance to one of the standard drugs on combined therapy. In all cases the bacilli were resistant to all but one of the drugs before the start of treatment, although this information was not available at that time. The organisms were fully sensitive to the remaining drug. The incidence of organisms of low degrees of resistance to streptomycin, P.A.S. or isoniazid in these cases is compared in table 45 with that in the average resistant population. For streptomycin and P.A.S. the incidence is of the same order in both groups. /

groups. The number of cases of isoniazid resistance is too small to allow of a direct comparison, but it is to be noted that in two patients whose organisms were resistant to 0·2 and 1·0 µg. per ml. respectively pre-treatment, resistance to a second drug developed. It therefore seems that resistance on combined therapy can occur with low or high degrees of resistance.

Persistant sputum positivity.

The details of 34 cases in which the sputum remained persistently positive throughout treatment of at least 6 months' duration are given in table 46. The results are summarised in table 47. The incidence of organisms of a low degree of resistance in patients in whom the sputum remained positive during treatment was of the same order as that found in an average resistant population. Therefore chemotherapy failed to convert the sputum in spite of the presence of bacilli of only low degrees of resistance.

Clinical and radiological deterioration.

The reports of clinical and radiological deteriorations are those of the clinicians in charge of the cases. The details of the cases are given in table 48, and the results summarised in table 49. It will be seen that the incidence of bacilli of low degrees of resistance was of the same order in cases in which clinical or radiological deterioration occurred, as in the average resistant population.

Summary. /

Summary.

The results reported here suggest that organisms of a low degree of resistance to streptomycin, P.A.S. or isoniazid are as significant bacteriologically and clinically as those of higher degrees. Therefore the criteria for resistance adopted in the earlier part of this thesis are supported by the correlation with clinical experience.

DISCUSSION

A wide variety of methods have been reported for the detection of drug resistance in tubercle bacilli. By the time this work was started in 1953, the most common ones in use were serial dilution techniques in liquid medium with Tween 80 and in coagulated egg medium. The results were usually reported as the minimum inhibitory concentrations of the drug that would completely or partially inhibit the growth of the test organism, or as a ratio of the inhibitory level of the test organism to that of a standard sensitive strain.

As has already been pointed out by Rist et al. (1951) and Canetti (1955), the criteria to be satisfied before a strain was considered resistant varied greatly even when similar methods of testing and reporting were used (see also tables 1, 2 and 3). This has led to wide misunderstanding in the interpretation of resistance tests and also in the assessment of the efficiency of different chemotherapeutic regimes in preventing the emergence of resistant bacilli.

In the present series the results of tests carried out by the same techniques on pre-treatment cultures in different laboratories showed some variation when the results were expressed as minimum inhibitory concentrations. There was, however, good agreement when the readings were given as resistance ratios to the standard H37Rv strain. /

strain. This suggests that the variations, probably due to minor differences in technique and media, affect the control strain in a similar manner to the test organism and are therefore neutralised when the ratio is used. All results for streptomycin and P.A.S. resistance tests in this thesis have therefore been expressed as resistance ratios. The results of isoniazid resistance tests have been reported as the minimum concentration of the drug inhibiting the growth of 20 or more colonies. This method was that adopted by the Medical Research Council for the trials of chemotherapy in tuberculosis (1952b;1953d). Many of the cases used here were included in those trials, and the method was found to be quite satisfactory. This may be partly due to the stability of isoniazid in the medium (Goulding et al., 1952; Mitchison, 1952) and therefore to the easier reproducibility of results. The present series confirmed that the 20-colony end-point recommended by the Medical Research Council was the most satisfactory.

In interpreting the results of tests carried out by various methods, it is important first to test a series of strains from patients who have never received the drug. There are however occasionally cases whose organisms are resistant to one or more drug (Harold, 1951; Arany, 1952; Thomas et al., 1954; Murdoch and Grant, 1955; Fox et al., 1957; Mitchison and Selkon, 1957) before the start of treatment due to infection with resistant bacilli. Such cultures should be excluded from a pre-treatment series, since they have /

have in fact been in contact with the drug at an earlier time. In the present series three such strains had to be discarded.

From the results of pre-treatment cultures, it is possible to determine the lowest readings which are outwith the normal variation of sensitive strains for the conditions of the test under consideration. For practical purposes a result which will occur only twice in 100 strains has been considered to signify an increase in resistance beyond normal biological variation. It is realised that in so doing a few sensitive strains will initially be classed as of low degrees of resistance. A repeat teat on the same culture or the simultaneous testing of other cultures from the same patient will, however, differentiate the true sensitive strain from one of a low degree of resistance.

The aim of any resistance test designed for use in a clinical laboratory is the detection of all degrees of resistance that are of clinical significance. In assessing the value of the different methods it is therefore important to correlate the bacteriological findings with the clinical data of the patients. This has not been done in reported series. In the present work, the results obtained by the different methods have been compared. Where a discrepancy occurred, the details of the cases have been investigated in an attempt to ascertain which result was most closely related to the clinical findings.

The effect of the duration of incubation of both solid and liquid medium tests was investigated. In the liquid streptomycin tests, /

tests, a higher proportion of strains was found to be resistant after 14 days' than after only 10 days' incubation. This is in agreement with the work of Mitchison (1950a) who showed that the late appearance of resistance was correlated with the percentage of resistant bacilli present in the original culture. In the solid medium tests, a number of cultures failed to grow on the resistance test after 14 days' incubation. Therefore, although a provisional report of resistance could sometimes be given at this time, 28 days' readings were found to be more satisfactory for routine use.

Pyle (1947) showed that there were present in a sensitive strain small numbers of bacilli resistant to streptomycin. Similar findings were also made regarding isoniazid (Middlebrook, 1952). It is therefore reasonable to expect small numbers of colonies to occur on drug-containing slopes from sensitive strains. This has already been shown for isoniazid tests (Medical Research Council, 1953d).

Comparisons of the readings for streptomycin, P.A.S. and isoniazid resistance tests, taking no growth, 20 colonies and 100 colonies as the end-points respectively, have confirmed that up to 20 colonies may occur on drug-containing slopes. Therefore the "no growth" end-point was considered unsatisfactory for use. Little difference was found between the 20-colony and the 100-colony end-points. For the streptomycin and P.A.S. tests, the result were slightly in favour of the 20-colony end-point. Therefore, since uniformity is a definite advantage in a routine laboratory, the 20-colony end-point has been used for all solid medium resistance tests.

Having decided on the optimum criteria for streptomycin and P.A.S. resistance for each of the two media, the results by the two methods were compared. Tests carried out in both media simultaneously showed that the solid medium was more efficient in detecting streptomycin and P.A.S. resistant organisms than was the liquid medium with Tween 80. These findings are in agreement with those of Holt and Cruickshank(1949) who showed that 15 strains which had a resistance ratio for streptomycin of 2 to 4 in Dubos medium with Tween 80, were capable of growing on solid medium containing 30 µg. streptomycin per ml. Where discrepancies occurred between the two methods in the present series, the results of solid medium tests showed a closer correlation with the case histories.

Various workers have shown that the presence of Tween 80 in Dubos and Davis liquid medium enhanced the anti-tuberculous activity of streptomycin (Fisher, 1948b; Williston and Youmans, 1949). It was however suggested (Fisher, 1948b; Medical Research Council, 1948) that the addition of 0·3 percent bovine albumin to the medium partly neutralised the effect of the Tween, while still permitting of a dispersed growth.

The testing of some cultures in liquid medium with and without Tween 80 in the present series showed that the results in the medium without Tween were more closely related to the solid medium tests, and therefore to the clinical findings, than were those in liquid medium with Tween. This suggests, as would be expected from the work of Fisher (1948a) that the failure to demonstrate resistance/

resistance in the liquid medium with Tween was largely due to the presence of the Tween 80, in spite of the presence of the bovine albumin.

The main disadvantages of the solid medium tests commented on by Rist et al (1951) and Canetti (1955) are the lack of standardisation of the inoculum and the heating of the medium after the addition of the drug. With regards to the inoculum, it has been shown that solid medium tests for streptomycin (Williston and Youmans, 1949), P.A.S. (Delaude et al. 1949; Rist, 1951) and isoniazid (Mitchison, 1952; Lecocq and Linz, 1952) are relatively unaffected by variations in inoculum between 10^4 and 10^6 bacilli. Further, many strains that are highly resistant to isoniazid grow poorly or not at all in semi-synthetic liquid medium (Fisher, 1952), thus making tests in this medium for any drug unreliable on such strains.

The heating of coagulated egg medium has been shown to cause a breakdown of streptomycin into inactive products (Drummond et al., 1951). P.A.S. (Drummond et al., 1951) and isoniazid (Mitchison, 1952; Goulding, 1952) would appear to be more stable. When a breakdown does occur, it may be variable and not accurately estimatable. If however the results are expressed as resistance ratios to a standard strain, a culture of which is included in each batch of tests, the actual concentrations of the drug in the tubes inoculated from the test and standard strains should be identical. The ratio will therefore be unaffected by any deterioration of the drug.

One /

One of the advantages of the solid medium method, apart from the greater efficiency in detecting resistant bacilli, is that contaminants are more readily detected by colonial characteristics than by the turbidity in liquid medium. Further, as pointed out by Rist et al. (1951) and Canetti (1955), an approximate assessment of the proportion of the population resistant to the varying concentrations can be made by noting the amount of growth present in each tube. The significance of such variations is discussed in Section II of this thesis.

In many of the methods recommended for testing for resistance in tubercle bacilli, readings just above the upper limits of normal pre-treatment strains are not considered to be resistant(D'Esopo and Steinhaus, 1947; Wolinsky et al., 1948; Bernstein et al., 1950; Medical Research Council, 1952b, 1953d). In order to determine the significance of these low degrees of resistance, various factors have been considered in the present work.

It has been shown that a rise in sputum positivity frequently occurs with the emergence of bacilli resistant to streptomycin (Crofton and Mitchison, 1948; Mitchison, 1950b), P.A.S. (Horne, 1949) or isoniazid (Joiner et al., 1952, 1953; Steenken et al., 1952; Coates et al., 1953; Collard et al., 1953; Medical Research Council, 1953c; Wallace et al., 1954; Widelock and Robins, 1954). No such rise is seen in patients whose bacilli remain sensitive to the drug (Medical Research Council, 1953c). In the present series the incidence of patients /

patients whose bacilli were of a low degree of resistance was as frequent in cases in whom an increase in the bacterial content of the sputum was demonstrated with the emergence of organisms resistant to streptomycin, P.A.S. or isoniazid as in the average resistant population. These results indicate that even when only a low degree of resistance was detectable, the drugs were ineffective in preventing the multiplication of the bacilli in vivo.

If adequate doses of combined drugs are given, and if the patient's organisms are sensitive to the drugs in use before the start of treatment, resistant bacilli will only emerge in a very small percentage of cases (Medical Research Council, 1953 a, b and c). If however the organisms are resistant to one of the drugs before the start of treatment, resistance to the second drug will probably develop as frequently as if the "sensitive" drug were being given alone (Turnbull et al., 1953; Medical Research Council, 1953c). In the present work, the development of resistance to a second drug was shown to occur with bacilli of both low and high degrees of resistance. This suggests that when a patient's organisms are of a low degree of resistance to a drug, that drug is likely to be as ineffective in preventing the emergence of resistance to a second drug as when the bacilli are highly resistant.

The Medical Research Council (1953c) showed that conversion of the sputum to negative by the end of six months' treatment occurred in 82 percent of patients receiving daily streptomycin and P.A.S. and in /

in 94 percent of those treated with daily streptomycin and isoniazid, provided that the bacilli were sensitive to both drugs at the start of treatment. Where resistance to one or both drugs is present, the sputum may never become negative (Medical Research Council, 1953c). The present work has shown that sputum positivity can persist regardless of the degree of resistance present.

In the reported series of cases treated with daily streptomycin in combination with isoniazid or P.A.S. (Medical Research Council, 1953a), clinical or radiological deterioration only occurred in 1 to 2 percent of the patients. In the present series, deterioration was reported while on treatment from a number of cases whose organisms had always been known to be of a low degree of resistance. This again suggests that the drugs were unable to control the infection even though only a low degree of resistance was detected.

To summarise, a comparison of the efficiency of resistance tests to streptomycin and P.A.S. in Dubos and Davis' liquid medium with Tween 80 and in coagulated egg medium has shown that the solid medium is the more sensitive test. Results by this latter method for streptomycin, P.A.S. and isoniazid are in close agreement with the clinical findings in the patients. Tests for the clinical and bacteriological significance of organisms of low degrees of resistance suggest that all degrees of resistance outwith the normal range of pre-treatment cultures are of importance.

SECTION II

STUDIES ON VARIATIONS IN DEGREE OF ISONIAZID RESISTANCE.

Outline.

Introduction and review of the literature.

Scope of present work.

Methods.

Results.

- Variations in the sputum.
  - Correlation with routine resistance tests.
  - Correlation with stage of development of isoniazid resistance in the sputum.
  - Effect of duration of isoniazid therapy.
  - Effect of time since last treatment with isoniazid.
  - Effect of further treatment with isoniazid.
- Variations within the lung.
  - Variations within individual cavities.
  - Variations between individual lesions.

Discussion and summary.

INTRODUCTION AND REVIEW OF THE LITERATURE

It has been shown that when bacterial resistance to streptomycin (Pyle, 1947; Mitchison, 1950b) or to isoniazid (Tompsett, 1954) occurs in patients suffering from pulmonary tuberculosis, organisms of varying degrees of resistance may be isolated from a single sample of sputum. Canetti and Säenz (1951) found that there may be variations in the degree of resistance of tubercle bacilli isolated from different lesions within the same lung. In all these reports, there were cases in which sensitive and resistant bacilli were demonstrated within the same specimen.

The presence of sensitive organisms in the lung and in the sputum of patients who are also harbouring resistant bacilli raises the question of the clinical significance of low percentages of resistant organisms. Canetti (1955) suggests, as regards isoniazid, that to withhold treatment with the drug from such patients may be depriving them of an effective drug. On the other hand, if the low percentages of resistant bacilli are significant, the use of combined treatment in such cases may mean the loss for the patient of a second drug to which his bacilli were fully sensitive.

The source of these variations is not yet ascertained. It has been suggested that they may arise as a result of the development of mutants of varying levels of resistance (Demerec, 1948; Mitchison, 1951). The work of Canetti and Säenz (1951) suggested that the finding /

finding of bacilli of low degrees of resistance or of full sensitivity in the presence of more highly resistant bacilli may be due to resistance having reached different levels in the different lesions at the time the examination was made.

Variations in the degree of resistance of organisms in the sputum may also be a result of the reversion of the degree of resistance in the bacterial population. That such a reduction can occur in the degree of isoniazid resistance of organisms in the sputum after the end of treatment was shown by Nitti (1952), Ashino (1953) and Petit (1953 a and b). This reversion was thought possibly to be due to the multiplication of sensitive organisms which had lain dormant but viable in the presence of the drug (Schaeffer, 1954) or to a back-mutation from the resistant population (Barnett et al., 1953a). Some workers (Medical Research Council, 1954) have suggested that apparent reversion may be due to variations in the resistance test or in the sampling of the bacterial population rather than to a genuine reduction in the degree of resistance.

There have been no reports on the significance of either the presence of sensitive and resistant bacilli simultaneously in the lung or of the reversion of resistance in the sputum.

SCOPE OF THE PRESENT WORK

In an attempt to ascertain the significance of low percentages of isoniazid-resistant organisms in the sputum of patients with pulmonary tuberculosis, variations in the degree of isoniazid resistance in the bacillary population of the sputum have been estimated. These results have been correlated with previous isoniazid therapy and with the effect of further treatment. Variations in the resistance of cultures from different lesions in the same lung are reported. Observations on the reversion to lower degrees of resistance and to full sensitivity are also made.

METHODS

Isoniazid population studies.

The method consisted essentially of inoculating a series of Löwenstein-Jensen plates containing varying concentrations of isoniazid with serial dilutions of the sputum concentrate. A plate which did not contain any drug was included in each series in order to give the total viable count. The numbers of organisms resistant to the various drug concentrations were thus determined. Owing to the tendency of Myco. tuberculosis to clump, the colony counts represent "viable units" rather than individual organisms.

Preparation of plates:- Fourfold dilutions of isoniazid in sterile distilled water were added to Löwenstein-Jensen medium in the proportion of one part of dilution to 100 parts of medium to give final drug concentrations ranging from 64 µg. per ml. by fourfold dilutions to 0·06 µg. per ml. The isoniazid-egg medium mixture was then poured into 9·0 cm. Petri dishes, approximately 30 ml. being used for each plate. The plates were inspissated at between 75° and 80°C in a hot air inspissator for one hour. Before use, the plates were dried in an incubator at 37°C. A disk of sterile filter paper was then placed in the lid of the Petri dish to prevent any water of condensation from dropping onto the surface of the medium.

Preparation of the sputum dilutions:- A morning specimen of sputum, to which had been added an equal volume of 4 percent sodium hydroxide, was mechanically shaken with glass beads for ten minutes /

minutes at room temperature. The mixture was then kept at 37°C for twenty to thirty minutes, depending on the tenacity of the sputum. It was subsequently centrifuged at 2,500 r.p.m. for twenty minutes. After the supernatant fluid had been removed and the deposit made neutral to phenol red with 8 percent hydrochloric acid, approximately 25 ml. of sterile distilled water were added to the container and the mixture was re-centrifuged as before. The resulting concentrate was homogenised by shaking with 2 ml. of sterile distilled water in a mechanical shaker for ten minutes. A series of tenfold dilutions of the homogenate was then prepared from which the plates were inoculated immediately.

The contents of lung cavities were treated in the same manner as the sputum.

Inoculation of plates:- The plates were inoculated with pasteur pipettes which delivered 50 drops to the ml. Two plates of each drug concentration and two control plates were inoculated from each specimen on a flat sheet of plate glass. One drop of one of four consecutive sputum dilutions was placed on the surface of each of the four quarters of every plate. The actual dilutions of the sputum concentrate used depended on the degree of positivity of the sputum. In most cases, dilutions of 1 in 10 to 1 in 10,000 were used for the control plates and the undiluted homogenate and dilutions of 1 in 10 to 1 in 1,000 were used for the plates containing the drug. After inoculation the plates were left undisturbed at room temperature for 18 to 24 hours protected from sunlight. The plates /

plates were then inverted, the filter papers removed and paraffin wax was poured into the rim to prevent evaporation during incubation. The plates were incubated for six weeks at 37°C.

Recording of results:- Counts were made from each plate at the lowest dilution of the sputum giving discrete colonies. The numbers of organisms resistant to the various drug concentrations were expressed as percentages of the total viable population. The results of the population studies have been classified according to the following table:-

POPULATION STUDY GRADING	PERCENTAGE OF TOTAL COUNT RESISTANT TO ISONIAZID - µg./ml.						
	NIL	0·06	0·25	1·0	4·0	16·0	64·0
SENSITIVE	100	0·05	0	0	0	0	0
LOW VARYING	100	0·05-69	0-69	0-69	0-69	0	0
LOW UNIFORM a	100	70-100	70-100	70-100	0-100	0	0
or b	100	70-100	70-100	0-100	0	0	0
or c	100	70-100	0-100	0	0	0	0
HIGH VARYING	100	0·05-100	0-100	0-100	0-100	0-69	0-69
HIGH UNIFORM	100	70-100	70-100	70-100	70-100	70-100	0-100

Routine resistance tests.

The routine isoniazid resistance tests referred to in this section were carried out by the method referred to on page ..47 of Section /

Section 1. Sputum cultures were tested at 0·2, 1·0, 5, 10 and 50 µg. isoniazid per ml. Löwenstein-Jensen medium. The results were expressed as the highest concentration that allowed the growth of twenty or more colonies after 28 days' incubation.

Examination of lung specimens for variations in the degree of isoniazid resistance between lesions.

Specimens for examination were obtained at operation or post mortem.

Sampling of lesions:- The specimens were examined within six hours of operation or within twenty-four hours of the patient's death, except in one case (case 11) in which post mortem examination was delayed for seventy-two hours. The main sites of the disease were located by means of a recent radiograph. Samples were taken from each of these and also from a number of lesions which were not visible on the radiograph. Precautions were taken to minimise the spread of tubercle bacilli from one site to another by the use of separate sterile instruments for each lesion and by making small incisions through only part of the lung at any one time. The contents of cavities were removed. Caseous foci were cut out and macerated with scissors in a sterile Petri dish.

Isolation of tubercle bacilli:- The specimens were suspended in a small volume of sterile distilled water and an equal volume of 4 percent sodium hydroxide was added. After the addition of sterile glass beads, the mixture was shaken mechanically for ten minutes /

minutes and then incubated at 37°C. for twenty minutes. After centrifuging at 2,500 r.p.m. for twenty minutes, the supernatant was poured off and the deposit was made neutral to phenol red with 8 percent hydrochloric acid. Twenty-five ml. of sterile distilled water were added and the material was centrifuged as before. The supernatant was poured off. The deposit was inoculated onto three Löwenstein-Jensen slopes by means of a pasteur pipette. Cultures were incubated at 37°C. They were examined weekly.

Isoniazid resistance tests:- Isoniazid resistance tests were carried out on positive cultures from all lesions within two weeks of the first appearance of growth. If more than one culture from a lesion was positive the slope with the heaviest growth was used for the resistance test. If only a few colonies appeared, the growth was spread over the slope and was reincubated for a further two weeks. The cultures were tested by the method described for sputum on page .17. of section 1. The concentrations used were 0·2, 0·5, 1·0, 2·5, 5, 10, 25 and 50 µg. per ml. The tests were read after 28 days' incubation and the results were expressed as the highest concentration of the drug allowing the growth of 20 or more colonies.

Isoniazid treatment.

All the patients referred to in this section were treated with isoniazid in a dosage of 200 mg. daily with the exception of one (case V 30) who received 500 mg. daily.

RESULTS

VARIATIONS IN SPUTUM

Population Studies on patients who had never received isoniazid.

The results of population studies on sputum from 10 patients, who had never received isoniazid, are given in table 50. It will be seen that in only one (case X 6) of the 10 cases was any growth found on the lowest concentration tested, i.e. 0·06 µg. per ml. In that case only one in 4,000 of the organisms grew at this concentration, and no growth occurred at higher concentrations. In all the cases, the routine test showed that the strains were sensitive to 0·2 µg. isoniazid per ml., the lowest concentration tested.

Population Studies on patients who had received isoniazid therapy and whose organisms were known to have been resistant to the drug by the routine test.

The details of the results of the population studies together with the results of the routine resistance tests, details of previous isoniazid therapy and of previous sputum resistance tests in 43 cases are shown in table 51 and figure 4.

Comparison of the results of the population studies and those of the routine resistance tests. Table 52 shows the correlation between the results of the population studies and those of the routine resistance tests. The population study results are expressed /

expressed as the highest concentration allowing any degree of growth. The results of the routine resistance tests are given as the highest concentration allowing the growth of 20 or more colonies after 28 days' incubation. The figures within the diagonal squares represent those studies in which the results of both tests were in agreement. Those to the right of this diagonal are cases in which growth occurred at a higher concentration in the routine test than in the population study, and those to the left cases in which growth in the routine test occurred at a lower concentration. Since a two-fold variation is within the experimental error of the routine method, only those results which are at variance by more than one concentration are considered as discrepancies.

The analysis of the five cases which showed this dis-: crepancy is given in table 53, the detailed results of the growth on the routine test also being given. In two of the cases (cases V 13 and V 29) the routine test showed growth at higher concentrations than that on the population study. This may have been due to the picking off of an odd colony of a higher degree of resistance when the suspension for the routine test was prepared. These more highly resistant bacilli may have occurred too infrequently to be detected in the population study. The weight of growth on the drug-containing slopes in the routine test was high in both cases. Therefore it is unlikely that the growth at higher concentrations on the routine test is due to the detection of very small numbers of highly resistant bacilli /

bacilli from the heavier inoculum. In both of these cases the tests were carried out when previous serial routine tests on sputum had suggested that a mixed bacterial population might be present. (see page 65) In case V 13 resistance was just developing, and in case V 29 a reversion in the degree of resistance was taking place.

In two of the three cases (cases V 6 and V 17) in which growth occurred in higher concentrations in the population study than in the routine test, the percentage of the organisms resistant to the higher concentrations of the drug was only 0·04 and 0·02 percent respectively. In one of these cases (case V 6) reversion to full sensitivity had occurred in the sputum, and it is likely that the routine test was failing to detect the low numbers of resistant organisms. Although growth of more than 0·1 per cent of the total population at 0·06 µg. isoniazid per ml. is probably outwith the normal range in the population studies, this is not the case in the routine test (see section 1, page 24). In the second case, (case V 17) reversion to a lower degree of resistance was occurring, and the population study confirmed that only small numbers of highly resistant organisms were present. Therefore it is likely that in this case also the routine test failed to detect the low numbers of organisms resistant at the higher levels of the drug.

In the third case (case V 20), 10 percent of the bacilli were resistant to 4 µg. per ml. and 0·7 percent to 16 µg. per ml. on the population study, although the routine sputum tests had repeatedly /

repeatedly given a reading of 1·0 µg. per ml. It is possible that the more highly resistant organisms may have died out during sub-culture.

To summarise, of the 43 sputa tested, all but five showed a close correlation between the result of the two tests, although the percentage of growth at the maximum concentrations was less than 0·1 percent in some cases. In only one case was there any suggestion that organisms of a high degree of resistance had died out or been overgrown by the more sensitive bacilli during the primary culture on Löwenstein-Jensen medium. Nevertheless the population study may be a slightly more sensitive test for detecting small numbers of bacilli of low degrees of resistance than is the routine test.

Correlation between the results of population studies and the stage of development of isoniazid resistance in the sputum as shown by routine sputum resistance tests.

The possible results of serial isoniazid resistance tests on cultures from the sputum of a patient during treatment with isoniazid alone and subsequent periods off the drug is shown in figure 5. Initially the organisms will be sensitive to the drug (1). As resistant organisms emerge, an increase in the readings will be observed (2). In the majority of cases the bacilli untimately become resistant to 50 µg. per ml. (3a), but in some patients the bacilli never grow at concentrations above 1·0 µg. per ml. (3b). If treatment with the drug is stopped, the bacilli may retain /

retain indefinitely the high or low degree of resistance reached by the end of treatment. In such cases the organisms are said to have remained at a "high" (3a) or "low" (3b) plateau of resistance respectively. In some cases, however, a reversion (4) to lower degrees of resistance (5) or to full sensitivity (6) may occur.

In table 54 the results of the population studies, on the sputa from 52 patients, grouped according to the classification given on page 59, are correlated with the stage of resistance as shown by routine resistance tests on successive cultures isolated from the sputum up to the time the population study was carried out (figure 4).

None of the 10 cases whose bacilli were sensitive to the drug had ever received isoniazid therapy. All of the seven tested at the time that resistance was first developing had mixed populations. Eleven cases had shown a high level of resistance for 3 to 25 months. In four of these (cases V 31, V 36, V 42 and V 45) all the organisms were of a high level of resistance. In the remaining seven cases, there was a mixed population, including some highly resistant bacilli. All the organisms were found to be of a low degree of resistance in five of the nine cases in which the sputum cultures had remained at a "low" plateau and had never shown a high degree. Three of the remaining four cases had a mixed population, but did not contain any highly resistant bacilli. In the ninth (case V 20; table 51) only 0·7 percent of the organisms were resistant to 16 µg. per ml.

Only one case (case V 28) was tested while reversion to

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a lower degree of resistance was occurring. The organisms in the sputum showed variations in degree, including bacilli of a high degree of resistance. Eight cases were tested in which reversion to a lower degree of resistance had occurred. In six of these all the organisms were of a low degree of resistance, no highly resistant bacilli having been detected. In the remaining two cases, the resistance of the bacilli varied. In only one of these (case V 21) were organisms of a high degree of resistance found. In this latter case, only single colonies grew on the higher concentrations of the drug. The assessment of the stage of development of resistance was made on only three recent resistance tests. It is possible therefore that the highly resistant organisms were present only irregularily in the sputum.

Finally sputa were tested from six patients whose routine resistance tests had shown reversion to complete sensitivity. Four of these were fully sensitive on the population study, no growth being obtained at 0·06 µg. per ml. The remaining two (cases V 5, V 6) contained some organisms of a low degree of resistance. The percentage of bacilli growing at 0·06 µg. per ml. was 15 and 0·3, and at 0·25 µg. per ml., 0·02 and 0·04 respectively. Therefore in these cases reversion to complete sensitivity had not occurred.

<u>Effect of duration of isoniazid therapy on the population study result</u>.

By the time this work was started, it was known that in

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a high percentage of cases of pulmonary tuberculosis the bacilli would become resistant to the drug if the patients were treated with isoniazid alone. It was therefore not ethical to treat patients in this way. In many of the cases on whom the population studies were carried out, the previous treatment with isoniazid had consisted of more than one course of the drug with breaks of varying periods. In only five cases was the population study carried out when the patient had been on isoniazid continuously since the first development of resistance. The results of these studies together with the duration of isoniazid therapy are given in table 55. In this small series there is no correlation between the duration of therapy and the pattern of resistance in the population study. Although there are only so few cases, the variations are so great that it seems unlikely that with larger numbers any correlation would be shown.

Effect of the duration since the last treatment with isoniazid on the results of the population studies.

In order to avoid the complications of repeated periods on and off the drug, this correlation has been limited to the seven cases who received a single course of the drug, followed by a single continuous period off isoniazid. The results are shown in table 56. It will be seen that in all of the five cases who had received less than seven months treatment (cases V 2, V 6, V 11, V 19 and V 33) the organisms had been shown to be highly resistant by the routine /

routine test during treatment. In three of these (cases V 2, V 6, and V 11) the routine test had shown reversion to sensitivity, though this was confirmed by the population study in only one case (case V 2). The fourth case (case V 19) showed reversion to a low degree of resistance by the routine test and the population study. These four cases had been off treatment for 17, 10, 20 and 15 months respectively. In the fifth, (case V 33) no reduction was detected. This patient had only been off treatment for six months.

The remaining two cases (cases V 9 and V 17) had received treatment for 14 and 17 months respectively. They had been off the drug for 33 and 26 months by the time the population study was carried out. In the first (case V 9) reversion to sensitivity had occurred in the sputum, though organisms of a low degree of resistance were detected on the population study. In the second case reversion to a lower degree of resistance had also occurred.

It would therefore seem that, even after prolonged treatment, reversion to a lower degree of resistance and even to full sensitivity may occur although a high degree of resistance was found during treatment.

There seemed to be no correlation between the therapy on which resistance developed and the degree of resistance to which the strains subsequently reverted. In all but one of the cases (case V 17) reversion to a lower degree of resistance occurred while the patient was either off all therapy or while receiving drugs /

drugs to which his organisms were known to be resistant. In case V 17 the patient was receiving pyrazinamide, tetracycline and streptomycin, but her bacilli were resistant to streptomycin and pyrazinamide.

Effect of further treatment with isoniazid in cases on whom population studies had been carried out.

Further treatment with isoniazid alone or in combination with other drugs, or with isoniazid derivatives that were known to act as isoniazid, was given to eighteen of the cases on whom population studies had been carried out. Table 57 shows the percentage of the organisms resistant to the lower concentrations of isoniazid together with the details of the subsequent treatment given, sputum positivity before and during treatment and the results of resistance tests to isoniazid and to a second drug, where applicable, before and after treatment.

In only two cases (cases V 2 and V 4) were the organisms shown to be sensitive both by the population studies and by the routine test. In one of these (case V 2) treatment with daily streptomycin, P.A.S. and isoniazid was given for one and a half months, at which time the patient left hospital against medical advice. The bacilli were known to be resistant to both streptomycin and P.A.S. before the start of treatment. The sputum, which was only positive on culture before treatment, converted to negative. No change in the isoniazid resistance occurred during this short period. Later, however, the patient returned to hospital with a strongly /

strongly positive sputum. He was treated with daily P.A.S. and isoniazid. There was no effect on the sputum positivity, but the bacilli became resistant to 50 µg. isoniazid per ml. In the second case with isoniazid sensitive organisms on the population study (case V 4), treatment with daily P.A.S. and isoniazid was given, although the bacilli were irregularly resistant to P.A.S. before the start of treatment. A reduction in sputum positivity did occur but, with the emergence of bacilli highly resistant to isoniazid, the organisms again became numerous. A population study repeated at the end of treatment confirmed that the bacilli were again highly resistant.

In four of the cases (cases V 5, V 8, V 9 and V 12) in which the routine test showed a low degree of resistance, less than 70 percent of the population was resistant to 0·06 and 0·25 µg. isoniazid per ml. Three of these (cases V 8, V 9 and V 12) were treated with pyrazinamide and isoniazid daily. In one of these three (case V 9) there was a marked fall in sputum positivity followed by a rise when the bacilli became resistant to pyrazinamide; in only one (case V 12) did the degree of resistance to isoniazid increase by the end of treatment. No reduction in sputum positivity occurred in the third case (case V 8) treated with pyrazinamide and isoniazid, although the organisms later developed resistance to pyrazinamide. There was no alteration in the degree of resistance to isoniazid during treatment in this case. The fourth case (case V 5) whose sputum contained only bacilli of a low degree of resistance was treated with oxytetracycline, 5 g. daily, with isoniazid 200 mg. daily. The sputum, which had been strongly positive /

positive before the start of treatment, converted to negative, but ultimately became positive again with the emergence of bacilli highly resistant to isoniazid.

It seems therefore that although in the three cases treated with pyrazinamide, the effect on the sputum positivity was probably due to the pyrazinamide, in the fourth case there was a definite effect which could only be due to isoniazid. The ultimate failure of treatment in this case might well have occurred on the oxytetracycline-isoniazid treatment even if the organisms had been sensitive to isoniazid at the start of treatment.

Six cases (cases V 11, V 17, V 19, V 23, V 25 and V 29) were re-treated in whom the pre-treatment population study showed that all the organisms were of a low degree of resistance. Three of these cases (case V 17, V 25 and V 29) were treated with "Dipasic", an equimolecular chemical combination of P.A.S. and isoniazid. In none was there any effect on the positivity of the sputum. In only one of them (case V 17) was there an increase in the degree of isoniazid resistance during treatment. Two of the remaining three cases (cases V 11 and V 19) were treated with daily streptomycin, P.A.S. and isoniazid, although their bacilli were known to be resistant to streptomycin and P.A.S. before the start of treatment. In case V 11 no effect from re-treatment was demonstrated. In case V 19, the organisms were of a low degree of resistance to streptomycin and P.A.S. Streptomycin and P.A.S. population studies carried out before /

before the start of combined treatment showed that all the organisms were resistant to 16 µg. streptomycin per ml. and to 1·0 µg. sodium P.A.S. per ml. No growth was however obtained on a plate containing 16 µg. streptomycin, 0·25 µg. isoniazid and 1·0 µg. sodium P.A.S. per ml. simultaneously. After the start of treatment, the cultures became negative, but, while still on treatment, the sputum later became strongly positive with the emergence of bacilli highly resistant to isoniazid. In the sixth case (case V 23), treated with daily pyrazinamide and isoniazid, sputum conversion occurred, but the positivity returned with the emergence of pyrazinamide-resistant bacilli. Therefore in only one of these six cases was there any apparent effect from the isoniazid treatment, and in that case it might have been at least partly due to streptomycin and P.A.S.

Variations in resistance of the organisms in the sputum, including highly resistant bacilli, were demonstrated in six cases before re-treatment (cases V 21, V 28, V 33, V 40, V 41 and V 27). All but one of these cases were treated with "Dipasic"; the sixth case (case V 27) was treated with daily pyrazinamide and isoniazid. In no case was there any effect on sputum positivity as a result of re-treatment. All cases were highly resistant by the end of treatment.

VARIATIONS IN LUNG

Results of population studies on lung cavities.

The results of population studies on the contents of ten lung cavities from 8 patients are shown in table 58. It will be seen that the patterns of variation in resistance are similar to those found in the sputum.

Variations in the degree of resistance of cultures isolated from different lesions within the same lung.

Table 59 and figure 6[illegible] show the results of isoniazid resistance tests on cultures from 125 individual lung lesions from 13 patients. In the first nine patients it will be seen that the degree of resistance of cultures from the lesions lay within three consecutive concentrations of isoniazid. This variation is within the experimental limits for the method, and therefore in these patients the resistances can be considered to be uniform. In five of these cases (1, 2, 3, 5 and 6) the organisms were of a low degree of resistance, averaging 1·0 µg. per ml. and in one (case 4) the organisms were resistant to 5 µg. per ml. In the remaining three patients (cases 7, 8 and 9) with uniform resistance, the organisms were all highly resistant to isoniazid.

In four patients (cases 10, 11, 12, 13) the degrees of resistance in the lung showed a greater variation than could be accounted for by the method.

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The last culture tested from the sputum was of a similar degree of resistance to the maximum found in the lung lesions. In two (cases 11 and 12) of the four patients in which a variation in the degree of resistance was present within the lung, organisms of maximum degree of resistance were isolated from both cavities and caseous foci. In the other two cases, (cases 10 and 13) the maximum degree was found in the cavities in one and in the caseous foci in the other.

A comparison of the variations in degree of resistance of cultures from the lung lesions with the results of resistance tests on serial cultures from sputa to the time of the examination of the lung, based on the information in figure 6, is given in table 60. In 5 of the 6 cases in which reversion to a lower degree had been noted in the routine tests, cultures from all the lung lesions were of a low degree of resistance. A uniformly high degree of resistance in the lesions was found in three (cases 7, 8, 9) of the four cases (cases 7, 8, 9 and 12) examined when the sputum cultures had shown a high degree of resistance for 10, greater than 2, 5 and 16 months respectively. In two (cases 10 and 13) of the three cases in which resistance was developing or where reversion to a lower degree was occurring, variation was found in the resistance of cultures from the lung lesions.

DISCUSSION

Variations within strains.

The presence of small numbers of resistant mutants within a strain of tubercle bacilli sensitive to the drug was first demonstrated by Pyle (1947). She showed that in strains sensitive to streptomycin approximately one in 10^7 of the bacilli were resistant. Similar work with isoniazid-resistant bacilli (Middle-:brook, 1952; Szybalski and Bryson, 1952) revealed the presence of one resistant bacillus per 10^6 sensitive organisms. It was postulated that these resistant mutants could form the nucleus of a resistant strain which would develop in the presence of the drug, since they would then be at a biological advantage. Following the original work on streptomycin resistant strains, Pyle (1947) and Mitchison (1950b) showed that organisms of varying degrees of streptomycin resistance can be found within a single specimen of sputum from a patient whose organisms are resistant to the drug.

That strains resistant to isoniazid could also consist of bacilli of varying degrees of resistance was suggested by Fust and Böhni (1953) and Middlebrook and Cohn (1953) and was later shown by Tompsett (1954). Few studies were however carried out on the lines of those done by Pyle and Mitchison with streptomycin, since the greater control in the use of the drug and the avail-:ability of other drugs which in combinations with isoniazid prevented /

prevented the emergence of resistant bacilli made the use of isoniazid alone unethical. Therefore knowledge of the constitution of isoniazid-resistant strains is confined largely to investigations carried out when resistance has already developed.

The present work shows that although strains resistant to isoniazid by the routine tests may consist entirely of organisms of any given degree of resistance (within the limits of experimental error), they are commonly composed of bacilli of varying degrees of resistance. Occasionally only small numbers of the resistant bacilli are present, the remainder being sensitive to the drug. The amount of variation that may occur in the sputum is to a certain extent predictable by the examination of serial isoniazid resistance tests on sputum cultures.

These results have not added to the knowledge of the mode of development of isoniazid resistance. The presence of varying degrees of resistance in the sputum may, in a particular patient, be a reflection of variations between individual lesions or of variations within a single lesion, or both. Canetti and Säenz (1951) showed that tubercle bacilli with varying degrees of streptomycin resistance can be found within different lesions in the same lung; other workers (Armstrong and Walker, 1949; Dye and Standley, 1951; Medlar et al., 1951) have confirmed these findings. Similar variations have been found in the present series with isoniazid-resistant strains. The source of these variations may

be /

be due to a number of factors. Manthei et al. (1953) found that concentrations of isoniazid may vary in the lesions within the same lung. Singh and Mitchison (1954) showed that, when organisms were exposed to varied concentrations of isoniazid *in vitro* the higher the concentration of the drug the greater the degree of resistance of the surviving organisms. It is therefore possible that variations between lesions may be due to the development of bacilli of varying degrees of resistance in the lesions as a result of the contact of the organisms with differing concentrations of the drug. The variations in concentration may be due to varying penetration into the lesions, or to the breakdown of the drug into biologically inactive products at varying rates.

Canetti and Saenz (1951) also showed that cavitated lesions contained organisms of a higher degree of resistance than did solid foci. They suggested that this was due to the higher bacterial content of the open lesions, and the consequent higher mutation rate. The present results have not shown any consistently raised degree of isoniazid resistance in the cases examined.

It is not possible to use the sputum studies as a complete reflection of the developing resistance of a limited bacterial population, owing to these variations between lesions. Population studies on the contents of cavities in a limited number of cases has shown that variations of resistance can also occur within a single lesion, although such variations are not so marked as that often seen in the sputum. Only one lesion was examined during the first /

first course of treatment. In this case all the organisms were of a low degree of resistance. Two lesions examined from a patient whose bacilli had reverted to sensitive after the end of treatment contained only sensitive bacilli. This was the only case examined who had received a single course of treatment followed by a period off the drug. The remaining cases, who had had periods on and off the drug, showed varying degrees of heterogeneity.

It is possible that variations within a single lesion may be due to the presence of mutants of varying levels of resistance in the initial population, which will be able to multiply in the presence of the drug. Therefore the population may remain a mixture of varying degrees of resistance. When the drug is withdrawn, the highly resistant bacilli, which have been shown (Mitchison 1953b) to multiply less rapidly than those of lower degrees of resistance may be overgrown by the less resistant bacilli, or by sensitive organisms which may have lain dormant but unaffected by the drug (Schaeffer 1954). It is also possible that the highly resistant organisms may be less virulent than those of lower degrees of resistance and therefore more likely to die out in the absence of the biological advantage which they would have in the presence of the drug. In either case, a heterogeneity of resistance would occur for some time.

It might be thought that with prolonged therapy all the bacilli would develop a high degree of resistance. But it is possible that if low degrees of resistance are sufficient to protect the /

the organisms from the effect of the drug, such mutants would have no need to develop higher degrees of resistance. In the present series, only a few patients had received one continuous course of isoniazid prior to the examination of the sputum for variations in resistance. There was no suggestion that the pattern of variation of resistance found in the sputum was correlated with the duration of isoniazid therapy.

Clinical significance of low percentages of resistant organisms

There has been considerable discussion as to the clinical significance of low percentages of resistant organisms in the sputum. It has been suggested (Canetti, 1955) that small numbers of organisms of low degrees of resistance may not be of significance, and may even be ignored by the clinician. That these bacilli represent only a small proportion of the total bacterial population can be assessed by the use of solid medium routine tests, although such readings can only give approximate results, since the inoculum is not accurately standardised. The question however remains as to the use of such resultsto the clinician.

Of the 18 patients in the present series who were re-treated with isoniazid following the population studies, twelve were treated with the drug alone or in combinations with drugs to which the organisms were already resistant. In two of these in whom all the bacilli in the sputum appeared fully sensitive to isoniazid, the results of treatment on sputum positivity and on isoniazid resistance were /

were similar to those expected during treatment with isoniazid alone in patients whose organisms were sensitive to the drug (Medical Research Council, 1952 a,b, 1953c). Only one other case of these twelve showed any suggestion of a possible effect from treatment. In the sputum of this case 73 percent of the bacilli were resistant to 0·06 µg. isoniazid per ml. and 28 percent to 0·25 µg. per ml. Although all the population was resistant to both streptomycin and P.A.S. at concentrations of 16 µg. and 1·0 µg. per ml. of Löwenstein-Jensen medium respectively, no growth occurred on medium containing 16 µg. streptomycin, 0·25 µg. isoniazid and 1·0 µg. sodium P.A.S. per ml. simultaneously. The bacilli were of a low degree of resistance to all three drugs, and it is possible that in this case there was additive or synergistic action on the combined therapy. This action was not however equivalent to that of the three drugs in combination on sensitive bacilli in man, since the patient's sputum later became strongly positive and his organisms developed high degrees of resistance to all three drugs.

Only six cases were re-treated with therapy under which the effect of the isoniazid could be truly assessed. Five of these received pyrazinamide plus isoniazid and one was given five grams of oxytetracycline daily with isoniazid. In a series of cases treated with pyrazinamide plus isoniazid (McDermott et al., 1954) in which the bacilli were sensitive to both drugs before the start of treatment, sputum conversion occurred in 90 percent of the cases. Isoniazid /

Isoniazid resistance was found in only one of the 55 cases treated. No pyrazinamide resistance tests were done. Although in the present cases a reduction in sputum positivity occurred in three of the five cases treated with pyrazinamide plus isoniazid this was probably due to pyrazinamide alone (Yeager et al., 1952; Schwartz and Moyer, 1953). The subsequent increase in positivity with the development of pyrazinamide resistance suggests that, in the presence of isoniazid resistance, the combination was less effective than in cases with sensitive bacilli. This was so in spite of the fact that, pre-treatment, three of these cases had only 6, 65, and 67 percent of bacilli resistant to the lower concentrations of the drug respectively. It is also interesting to note that the bacilli in these cases would have come within the range of isoniazid resistance which Perry and Moyes (1955) suggested would be of maximum sensitivity to pyrazinamide. Yet, as has also been shown by Donnerberg et al. (1957), the therapeutic effect was less than that reported in cases with fully sensitive organisms.

From these limited results, it would seem that the presence of small numbers of isoniazid resistant bacilli in the sputum of cases with pulmonary tuberculosis are usually sufficient to reduce the efficiency of combined therapy, although there may be some transient activity.

Reversion to drug-sensitivity.

Although the reversion of resistance in tubercle bacilli

by /

by the routine resistance test has been reported by many (Nitti 1952; Ashino 1953; Petit 1953a and b) it has been suggested (Medical Research Council 1954) that the apparent reduction in the degree of resistance of cultures from the sputum may be due to chance variations in the routine test or in the population being tested. In their series they found that, although a reduction in the degree of resistance occurred in some patients after the end of treatment, there were a number in which an increase of resistance was reported. They were handicapped by only having three-monthly sensitivity tests, and also by the fact that the patients concerned were under the care of physicians in many different centres. Therefore it would not be easy for a careful follow-up of the treatment given since the end of the original course of therapy to be made. Personal experience has shown that unless a detailed examination of case records is made, short periods of drug treatments, which may be sufficient to affect bacterial resistance, are often omitted from the case summaries.

The results of the population studies in the present series would suggest that in fact reversion does occur in some cases, and that although the commonest form is a reduction to a lower degree of resistance, occasionally reversion to complete sensitivity may take place. The correlations between the results of population studies and of the routine resistance tests show that in the large majority of cases, highly resistant organisms will be detected /

detected by both tests simultaneously. Therefore where a population study showed that all the organisms were of a low degree of resistance, and this is borne out by repeated tests on sputum cultures, it is reasonable to conclude that highly resistant organisms present on earlier routine tests are no longer present in the sputum. It might be argued that the lesions containing the organisms of a high degree of resistance have become walled off and are no longer contributing to the sputum. But cultures from individual lesions in a case in whom this reversion in resistance had been shown to have occurred in the sputum before the lung was examined (see Table 59) have confirmed that in no major lesion were highly resistant bacilli present. It therefore seems proof that reversion to a lower degree of resistance and even to full sensitivity can occur. As has been pointed out above, this is more likely to occur after long periods off the drug.

If reversion is a back-mutation, it might be expected that it would occur most frequently when the bacilli were multiplying rapidly, that is during periods when the patients were untreated or receiving treatment which was not reducing the rate of multiplication of their organisms. In the present series, no reversion was seen during a period of treatment with effective drugs to which the organisms were sensitive, but there are insufficient cases to establish whether in fact such treatment would have any effect on the reversion rate.

If /

If reversion to full sensitivity occurs in a patient, it would be of importance to know if the bacilli would react to re-treatment in a similar way to organisms which had never been in contact with the drug. This is difficult to assess. If isoniazid, to which reversion has occurred, is combined with a standard drug, such as P.A.S. or streptomycin, to which the organisms are still sensitive, failure to protect against the development of resistance to the standard drug would demonstrate that reversion was of no clinical significance. But in such a case, the patient would have lost a valuable therapeutic weapon. If, however, isoniazid is combined with standard drugs to which the organisms are known to be resistant, it is probable that the patient is in effect being treated with isoniazid alone. Such treatment might be expected to fail in many cases, and isoniazid resistance would be likely to emerge in four to six weeks after the start of treatment. Therefore probably the most informative treatment would be five grams of oxytetracycline daily with isoniazid. This was shown to delay the emergence of isoniazid resistance in fully sensitive cases (Stewart et al. 1954) and to reduce the positivity in 5 of the 10 cases reported, but would not be expected to have any effect if the organisms were resistant to the drug, since oxytetracycline has little or no effect per se (U.S. Veterans Administration, 1951; Pfefer et al. 1952). Unfortunately this treatment was not used on the two cases with complete reversion reported here. It was however used on a case in which reversion had occurred on the routine /

routine test although 15 percent of the population was still found to be of a low degree of resistance by the population study. In this case resistant organisms reappeared in the routine test two and a half months from the start of treatment after temporary sputum conversion. This sort of result might have been expected with this combination of treatment. However, in the three cases reported (Stewart et al. 1954) who received 5 g. of oxytetracycline with isoniazid and in whom isoniazid resistance developed, this was not detected until at least the fifth month of treatment. Therefore in the case reported here, resistant organisms emerged earlier than in those cases whose bacilli were fully sensitive at the start of treatment. It is not possible to determine from these findings whether reversion to complete sensitivity, by the routine test and population study, does in fact render the bacterial population as susceptible to isoniazid as if the patient had never received the drug.

It has been suggested (Barnett et al. 1953a) that reversion may occur as a result of the overgrowth of resistant organisms by those of a lower degree of resistance. Mitchison showed that bacilli of low degrees of resistance or full sensitivity grow more rapidly than those of a high degree of resistance (Mitchison 1952). In the presence of the drug the resistant organisms would be at a biological advantage, but when the drug is withdrawn the more rapid growth rate will give the advantage to bacilli of a lower degree of resistance. It is not possible from the results of /

of the present study to draw any conclusions as to the mechanism of the reversion of isoniazid resistance in tubercle bacilli. The presence of varying degrees of resistance in the sputum of patients whose organisms are known to be reverting could be due to the occurrence of reversion irregularly within the descendents of resistant bacilli, or to the gradual overgrowth of resistant bacilli by more sensitive ones, either diffusely throughout the lung or in certain lesions.

In conclusion, these investigations into isoniazid-resistant bacillary populations in lung lesions and in sputum have shown that there may be wide differences in the composition of such populations. A follow-up of patients who were re-treated with isoniazid after the population study was carried out suggests that where only a low percentage of the bacilli are resistant an effect on sputum positivity may occur, but it will probably only be transient. It has been shown that reversion to lower degrees of resistance or to sensitivity can occur although it was not possible to determine whether this enabled the patient to obtain full benefit from further treatment with isoniazid.

SECTION III

SOME OBSERVATIONS ON THE VIRULENCE OF ISONIAZID - RESISTANT TUBERCLE BACILLI

Outline.

Introduction and review of the literature.

Scope of the present work.

Methods.

Results.

- Size of inoculum and virulence.
- Correlation of animal virulence with the pattern of isoniazid resistance in the sputum.
- Correlation of catalase activity with guinea pig virulence.
- Comparison of the degree of isoniazid resistance in cultures from the sputum and from the guinea pig spleen.
- Analysis of avirulent cultures.
- Virulence tests on strains from three patients infected with isoniazid-resistant bacilli.

Discussion and summary.

INTRODUCTION

There have been a number of reports of variations in the virulence for laboratory animals of strains of tubercle bacilli which have never been in contact with anti-tuberculous agents (Pätiälä, 1948; Stewart, 1951; Steenken and Wolinsky, 1953). In the series of Pätiälä and of Steenken and Wolinsky all of the strains from untreated patients caused some dissemination of disease although in many cases the virulence was markedly reduced. In contrast to these results many workers (Barnett et al., 1953a, b; Middlebrook and Cohn, 1953; Steenken and Wolinsky, 1953; and others) found that strains that were of a high degree of isoniazid resistance were often completely avirulent for guinea pigs.

Most of the original work was carried out on cultures which had either been made resistant in the laboratory (Barnett et al., 1953a; Middlebrook and Cohn, 1953) or which had been grown on medium containing isoniazid prior to the inoculation of the guinea pig (Barnett et al., 1953b; Peizer et al., 1954). These strains would be expected to be of a more uniformly high degree of resistance than those freshly isolated from patients.

In some of the reports the strains used were not cultivated in the presence of isoniazid before animal inoculation (Bloch et al., 1953; Middlebrook and Cohn, 1953; Peizer et al., 1953; Steenken and Wolinsky, 1953; Morse et al., 1954). In these series, the correlation between the degree of isoniazid resistance of the strains /

strains and the virulence for guinea pigs was less marked than in the series in which artificially - induced strains were used. The variable virulence in these latter cases was probably due to the heterogeneous nature of the strains (Morse et al., 1954; Peizer et al., 1954), the dissemination of the disease being due to the less highly resistant bacilli. Cultures from patients are likely to contain bacilli of low degrees of resistance which have been shown by Mitchison (1954) to be virulent for laboratory animals. Further, Meissner (1953, 1954) demonstrated that when mixtures of resistant and sensitive organisms were injected into guinea pigs, the bacilli isolated from the disseminated lesions were often fully sensitive to isoniazid, although those from the site of inoculation were of the same degree of resistance as the original resistant strain.

Certain *in vitro* tests have been correlated with the virulence of strains for laboratory animals. The loss of animal virulence was found to be associated with a decrease in the catalase activity of the bacilli (Middlebrook, 1954; Cohn et al., 1954). It was also suggested that the formation of serpentine cords in liquid medium was an indication of the virulence of the strain (Middlebrook et al., 1947; Dubos, 1948).

The question arises, from these reports of strains of tubercle bacilli with reduced virulence for laboratory animals, as to whether such strains are of reduced virulence for man.

SCOPE OF THE PRESENT WORK

In the present work the animal virulence has been correlated with the resistance pattern of the bacilli in the patient's sputum. In order to avoid any alteration of the resistance pattern in the inoculum during sub-culture, the sputum concentrate was inoculated into the guinea pig. Population studies, including a viable count, were made on an aliquot of the concentrate. In this way any apparent diminution in virulence due to a low inoculating dose would be detected. Furthermore the proportions of the bacilli in the inoculum resistant to the varying concentrations of isoniazid were assessed. In order to ascertain whether the dissemination was due only to organisms of a low virulence, cultures were made from the individual lesions post mortem and these were tested for isoniazid resistance. Routine isoniazid resistance tests and tests for catalase activity and cord formation were also made on cultures from the sputum concentrate.

METHODS

Guinea pig virulence test.

The sputum was concentrated by the method described for the population studies (see page 57.). The homogenate was prepared in 2·0 ml. sterile distilled water. 1·0 ml. of this suspension was inoculated into the left thigh muscle of the guinea pig. Isoniazid population studies were made on part of the homogenate, and the remainder was used to inoculate two Löwenstein-Jensen cultures.

In the three patients who had been apparently infected with isoniazid resistant bacilli, a culture on Löwenstein-Jensen medium was used for the virulence test. Approximately 0·5 mg. wet weight of a 14 to 21 days' growth was injected into the left thigh muscle.

Mantoux testing of guinea pigs. Three to four weeks after inoculation, 0·1 ml. of a 1 in 25 dilution of a solution of protein-purified derivative of mammalian tuberculin containing 2 mg. tuberculin per ml. was inoculated intra-dermally 24 hours after depilation. The diameter of induration at right angles to the needle track was read after 48 hours.

Post mortem examinations:- The animals were killed eight weeks after inoculation. Post mortem examinations were carried /

carried out on any animals that died earlier. The degrees of positivity of the inoculation site, inguinal and lumbar glands, spleen, liver and lungs were assessed macroscopically. They were graded as follows:-

		+++	++	+
Inoculation site abcess	-	2 cm. or more	1 cm.	0·5 cm. or less
Glands	-	1 cm. or more	0·5 cm.	Less than 0·5 cm.
Spleen	-	Approx. 4 x 2 cm.	Approx. 2 x 1 cm.	Scanty tubercles and no marked enlargement.
Liver and lungs	-	Numerous tubercles	Scanty tubercles in all lobes	Scanty tubercles in one lobe

The virulence of the strains was graded as follows:-

Fully virulent - Widespread dissemination in all lesions

Moderate virulence - Dissemination limited to two of the three organs examined, or positivity of the spleen only "+".

Low virulence - Only one organ involved.

Avirulent - No macroscopical dissemination.

Cultures were prepared from all the lesions with macroscopic disease by the same method as that used for the culturing of bacilli from the lung lesions (see page 60. section II). The glands, spleen, lungs and liver were macerated with scissors before treatment with the concentrating agents. Cultures were set up in duplicate.

Isoniazid resistance tests:- Cultures from all positive lesions /

lesions were tested for isoniazid resistance by the method described for sputum on page ..17.. . The concentrations used were 0·2, 1·0, 5, 10 and 50 µg. isoniazid per ml.

Löwenstein-Jensen cultures prepared from the sputum concentrates were also tested for isoniazid resistance by the same method.

In vitro assessments of virulence.

The following tests were carried out on the Löwenstein-Jensen cultures from the sputum concentrates.

Cord Formation. A sub-culture from the primary growth on Löwenstein-Jensen medium was made into Dubos and Davis liquid medium with Tween 80 at pH 7·0 (see page ..12..). After 10 days' incubation at 37°C, approximately 0·1 ml. of the culture was transferred into approximately 3·0 ml. of the following medium (Dubos and Middlebrook, 1947) in a ¼ oz. screw-capped bottle.

Basal medium -

KH_2PO_4	1·0 g.
$Na_2HPO_4 \cdot 12H_2O$	6·3 g.
Asparagin	2·0 g.
$CuSO_4$	0·0001 g.
$ZnSO_4$	0·0001 g.
$CaCl_2$	0·0005 g.
$MgSO_4 \cdot 7H_2O$	0·01 g.
Ferric ammonium citrate	0·05 g.
Enzymatic digest of casein	- 2 g.
"Tween 80"	0·02%
Distilled water	2,000 ml.

pH adjusted to between 6·5 and 6·8.

The medium was sterilised by autoclaving at 10 lb. per sq. inch for 10 minutes. Before use 5 percent human serum was added.

After /

After incubation at 37°C. for 10 days, stained smears of the cultures were examined for the presence of cord formation.

The results were classified as follows:-

+ Tight cords present

± Cords formed but bacillary forms still readily detectable.

-ve No evidence of cording.

Catalase Activity.

A loopful of growth from a 14 to 21 days' culture on Löwenstein-Jensen medium was added to 0·5 ml. of a solution containing equal parts of 100 vols. hydrogen peroxide and 10 percent "Tween 80" in a 3 x ¼" test-tube. Twenty minutes after the addition of the culture, the tubes were examined for the presence of oxygen bubbles.

++ A continuous ring of bubbles round the meniscus.

+ Although bubbles had risen to the meniscus, no complete ring was present

± Bubbles on clumps of bacilli in tube.

-ve No bubbles discernible with the hand-lens.

In vitro tests to detect saprophytic strains.

In order to detect any strains of saprophytic Mycobacteria, the following tests were carried out on cultures from the sputum.

Aryl-sulphatase - 0·2 ml. of a 10 days sub-culture in Dubos and Davis medium with Tween, pH 7·0, was inoculated into 4·5 ml. of Dubos and Davis medium with Tween 80, pH 7·0, to which had been added 0·2 ml. 10 percent bovine albumin fraction V and 0·5 ml. of 0·01 M seitz-filtered solution of phenol-phthalein disulphate. After incubation at 37°C for 14 days, 2N NaOH was added to the cultures drop by drop. The production of a red colouration indicated the presence of free phenol-phthalein.

Colony /

Colony type on solid medium. Plates were prepared in 3 cm. Petri dishes containing the following solid medium:-

Basal medium - As given for the tests for cord formation (see above) excluding the Tween 80 and serum albumin, but with the addition of 1·5 g. soluble agar. The oleic-acid-albumin complex was prepared by dissolving 0·12 ml. oleic acid in 10 ml. N/20 NaOH. 5 ml. of this solution were added to 95 ml. of a neutral 5 percent solution of bovine albumin fraction V in 0·85 percent saline. The solution was sterilised by seitz filtration. 10 ml. of the oleic-acid-albumin compound were added to 95 ml. of the base.

After incubation at 37°C. for 14 days, the colonies were examined under a plate microscope.

Resistance to thiosemicarbazones. Although it has been suggested that this test might be of use in differentiating between pathogenic and saprophytic strains of Mycobacteria, it had to be discarded in the present series, since the bacilli from a number of patients who had been treated with thiosemicarbazones at some time appeared to be resistant to the drug. In other tests these strains behaved as true Mycobacterium tuberculosis.

RESULTS

Table 61 gives the detailed results of the population studies on the sputa from 21 patients together with the results of tests for isoniazid resistance, catalase activity and cord formation on cultures from the same specimens. The results of the guinea pig virulence tests and of isoniazid resistance tests on cultures from the sites of disease in the animals together with the mantoux reaction 3 to 4 weeks after inoculation are detailed in table 62. Only one specimen is included from each patient. The results given in the tables are further analysed and discussed below.

Effect of size of inoculum on guinea pig virulence.

A comparison of the size of inoculum, as expressed by the viable count per ml. of sputum concentrate, and the virulence for the guinea pig for the 21 strains tested is given in table 63.

Sixteen of the strains were of moderate or high virulence for the guinea pig, although in three of these the inoculating dose was less than 10^4 bacilli. Of the five remaining strains, two (G 2 and G 8) were of low virulence and three (G 15, G 16 and G 18) were avirulent. In the two cases with low virulence, the infecting doses were 1×10^3 and 6×10^3 bacilli respectively. A second specimen collected from each of these patients, within two days of /

of that reported, was also tested for virulence. In one of the cases (case G 2) the repeat specimen was also of low virulence in spite of an infecting dose of 3×10^4. The second strain from the second case (case G 8) was of full virulence although only 6×10^2 organisms were inoculated. Therefore only one of these two strains was of diminished virulence.

Further specimens from two (cases G 15 and G 16) of the three cases in which avirulence was demonstrated for the guinea pig were also avirulent in spite of inoculating doses of 10^4 and 10^6 respectively. In the third case (case G 18), the inoculum of the repeat specimen was only 5×10^2. This strain again appeared avirulent. In this strain, the lack of virulence may have been due to the small numbers of bacilli inoculated, though it should be noted that three strains (G1, G 11 and G 19) showed moderate or high virulence in spite of an inoculating dose of less than 10^4 organisms.

Therefore although a low inoculum might have accounted for lowered virulence in one case, two of the strains with a high inoculum were also avirulent for the guinea pig.

Correlation of the results of animal virulence tests with the pattern of isoniazid resistance in the sputum.

A summary of the results of the population studies correlated with those of animal virulence tests is given in table 64. It will be seen that all the strains that were avirulent contained /

contained organisms of varying degrees of resistance, including some highly resistant bacilli. Strains showing a moderate degree of virulence tended to be more highly resistant than those that were fully virulent, although the numbers are too small for statistical analysis.

Correlation of catalase activity with guinea pig virulence.

The results of animal virulence tests and the catalase activity of the strains are compared in table 65. All of the three strains found to be avirulent in the guinea pig showed a reduction in catalase activity. All but two of the remaining 18 strains were of normal activity. The two exceptions were of moderate virulence.

Comparison of the degree of isoniazid resistance in cultures from the sputum and from the guinea pig spleen.

Table 66 shows a comparison of the isoniazid resistance of cultures isolated from the sputum and from the guinea pig spleen for 17 of the strains. In the remaining four cases (G2, G15, G16 and G18) cultures from the spleens were negative (see table 62). Allowing a one-tube variation as being inherent in a dilution technique, there was agreement within the experimental error between the two cultures in 14 of the strains tested. In each of the remaining three cases (cases G11, G13 and G19), the resistance of the culture from the spleen was of a lower degree

than /

than that from the sputum. In no case was the culture from the spleen of a higher degree of resistance than that from the sputum.

An analysis of the three cases in which the disseminated lesions in the spleen seemed to be caused by organisms of a lower degree of resistance than those predominating in the sputum is given in table 67. In two of these strains (cases G13 and G19) there was a variation in the degree of resistance in the bacilli in the sputum. In the first of these, (case G13) only 0·4 percent of the bacilli were highly resistant. Therefore it is possible that the dissemination occurred due to the more numerous, but less resistant, bacilli.

In the second case (case G19), 64 percent of the bacilli in the sputum were resistant to 16 µg. per ml. but the bacilli isolated from the spleen were only resistant to 1·0 µg. per ml.

In the third (G11) the inoculating dose of 450 organisms was very small. All the bacilli appeared to be resistant to 1·0 µg. isoniazid per ml., but the method is not sufficiently accurate to detect a small percentage of sensitive organisms in such a population. Nevertheless, if this were the case, the results would suggest that the resistant bacilli, present in overwhelming numbers, were less virulent than the few sensitive bacilli. It was not possible to attempt to reproduce these results, as the patient was operated on shortly after the examination was made and his sputum converted to negative immediately afterwards.

Analysis /

Analysis of avirulent cultures.

Table 68 shows a further analysis of the three strains which were avirulent for the guinea pig. It will be seen that in each case the avirulence for the guinea pig was confirmed on at least one further specimen of sputum. In none of these cases were the organisms all highly resistant to isoniazid, although it was not known whether they had been so at some time. All the three patients were receiving a second course of isoniazid, or an isoniazid derivative, at the time the specimen was examined and in each case this course had lasted for at least 19 months. Of the 18 cases whose strains were virulent, 10 had received treatment including isoniazid for 2 to 11 months immediately prior to the examination of the sputum and two had received 21 and 31 months treatment respectively. Of the remaining 6 cases, four had not been treated with the drug for 11 to 55 months, in one details of treatment were not available; the sixth case was infected with isoniazid - resistant bacilli.

In all three cases with avirulent strains there was a depression of catalase activity, but cord formation was demonstrated. In every case the aryl-sulphatase tests were negative and the colonial formation on solid Dubos was typical of strains of Myco-tuberculosis.

Clinical fate of the three patients whose strains were avirulent.

All of the three patients whose strains were avirulent for the guinea pig had extensive chronic disease with bilateral cavitation. /

cavitation. Following the examinations of the sputum reported above they were treated with isoniazid or with isoniazid derivatives. No clinical or radiological deterioration has occurred to the present time. However, the clinicians concerned consider that the lack of deterioration is consistent with the chronic fibro-caseous type of disease, and that this does not therefore necessarily indicate the presence of bacilli of low virulence for man.

Virulence tests on strains from three patients infected with isoniazid-resistant bacilli.

Table 69 shows the results of animal virulence and isoniazid resistance tests on three strains from patients who were infected with isoniazid-resistant bacilli having never previously received the drug. It will be seen that two of these strains (cases G22 and G23) both of which were resistant to 50 µg. isoniazid per ml., were avirulent for the guinea pig. The third strain (G 24) was of moderate virulence and a low degree of resistance. In all cases the Mantoux test at three weeks was positive. The catalase activity was reduced in the two avirulent strains.

DISCUSSION

It has been shown in section II of this thesis that strains of tubercle bacilli resistant to isoniazid isolated from patients treated with the drug in a dose of 200 mg. daily usually consist of organisms of varying degrees of resistance. That such strains give less consistent results on animal virulence tests when these are correlated with the degree of resistance of the original strain has been pointed out by Morse et al. (1954) and Peizer et al. (1954). That dissemination of disease following the inoculation into guinea pigs of cultures containing both resistant and sensitive bacilli can be due to the sensitive organisms has been demonstrated by Meissner (1953, 1954) and Karlson and Ikerni (1954). Direct cultures from such mixtures would show a high degree of resistance. But cultures from the disseminated lesions were sensitive, although those from the site of inoculation were highly resistant. Similarly, with mixtures of bacilli of low and high degrees of resistance, the dissemination may only be due to the organisms with low degrees of resistance. Other factors may however complicate the results.

First, it is possible that the more highly resistant bacilli may be present in the spleen although they may not multiply there. The organisms of lower resistance might have given rise to the macroscopic lesions, but cultures from the organ might appear highly resistant due to the isolation in vitro of the highly resistant /

resistant bacilli. The second factor affecting the correlations of virulence and resistance in mixed populations is that dissemination of the disease due to organisms of low degrees of resistance may occur by chance if these organisms are more numerous in the population. The cultures from the original sputum are still likely to appear highly resistant.

Therefore the final tests of virulence of strains from patients can only be made on strains of a uniform high resistance to isoniazid. It would also be necessary to prove that the disease in the organs was due to highly resistant bacilli. As has already been shown, uniformity of high degrees of resistance is not found very frequently in sputum from patients with pulmonary tuberculosis treated with isoniazid in doses of approximately 200 mg. daily, even though the sputum tests show a high degree of resistance. In no case in the present series tested for animal virulence were 100 percent of the organisms of a high degree of resistance. Therefore it was not possible to assess the virulence of such a strain.

In four of the eight cases in which highly resistant bacilli were isolated from the sputum, cultures from the disseminated lesions were also highly resistant. But since organisms of low degrees of resistance were also known to be present in these cases, the development of macroscopic lesions cannot with certainty be attributed to the highly resistant bacilli.

None /

None of the three avirulent strains were of a uniformly high degree of resistance on the population study, although in two the sputum cultures and cultures from the inoculation sites in the guinea pigs were both highly resistant. The evidence on these strains therefore suggests that the low virulence was common to organisms of all degrees of resistance, and that it was not confined to the highly resistant bacilli. There was no evidence that the low virulence was due to a low inoculum.

It has been suggested that the catalase activity of tubercle bacilli is correlated with the loss of animal virulence (Middlebrook, 1954; Cohn et al., 1954). In the present series diminished activity was found in the three avirulent strains and in two strains with moderate virulence. Cord formation (Middle-:brook et al., 1947; Dubos, 1948) was present in both virulent and avirulent strains.

The question arises as to whether strains of tubercle bacilli avirulent for laboratory animals might also be avirulent for man. If this were the case, it might be an advantage from the epidemiological aspect to induce such strains in patients who could not be made sputum negative (Oestreicher et al., 1955), since it would reduce the spread of the disease. The findings of avirulent strains in patients who have been infected with isoniazid resistant bacilli, and the low incidence of avirulence for animals in strains isolated from patients whose organisms were known to be highly resistant, would suggest that such an advantage is /

is unlikely to be found in clinical practice if doses of 200 mg. isoniazid daily have been used. It has not however been possible to confirm this in the present series. These cases were however not receiving high doses of isoniazid and therefore the avirulent strains referred to in the work of Oestreicher et al. (1955) were not likely to develop.

If the isoniazid-resistant strains are not of reduced virulence for man, an increase in the number of patients harbouring isoniazid-resistant bacilli might lead to a greater incidence of sputum-positive cases and possibly therefore to increased risks of infection in the community. Treatment of such cases of primary infection with isoniazid in combination with streptomycin or P.A.S. might lead to the development of resistance to streptomycin or P.A.S. in cases in which such combinations with fully sensitive organisms would normally bring about sputum conversion and probably complete sterilisation of the lung lesions (Stewart et al., 1956).

In conclusion, these investigations have shown only a low incidence of avirulent strains in a series of patients treated with low doses of isoniazid (200 mg. daily), chosen at random, the only criterion being that their organisms were resistant to isoniazid. The two cases of primary infection in man with strains that were avirulent for guinea pigs suggest that even though strains are avirulent for laboratory animals, they may nevertheless be capable of causing disease in man.

FINAL DISCUSSION

The object of a bacterial resistance test in a clinical laboratory is to detect organisms which are capable of multiplying in the presence of a chemotherapeutic agent. When a patient's bacteria are reported by such a test to be resistant to a drug, further treatment with that drug would no longer be expected to be effective in controlling the disease. But even when no further benefit is being derived from the drug, the patient's defences may be capable of dealing with the residual population, so that clinical failure is not always obvious. Such cases may cause confusion in the interpretation of the clinical significance of drug resistance. It is therefore perhaps preferable to consider the problem in another way, namely that if the organisms are re-:ported as sensitive to the drug, that drug should be clinically effective in every case.

There are a number of methods available for the demon-:stration in vitro of the presence of resistant organisms. Within each method there are also variations in technique, which may significantly alter the results. If the method to be used is decided solely on the bacteriological data, the bacteriologist will have no means of deciding which of these variations is the most efficient from the clinical stand-point, and may therefore choose a technique and criteria for resistance which would fail to detect some of the levels of resistance that are of clinical significance.

In the past most tests for drug resistance have been evolved largely as a result of bacteriological considerations and significant /

significant levels of drug-resistance have often been arbitrarily decided on such criteria as resistance to the highest drug level detectable in the blood serum of patients on customary dosage. As far as is known no previous attempt has been made to establish criteria of drug resistance which are closely correlated with clinical failure to respond to treatment with the drug. Such a study has required close co-operation between bacteriologists and clinicians but it is submitted that the levels of drug sensitivity suggested in this thesis are now well correlated with the only criterion of vital importance to the patient - successful arrest of the disease.

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A P P E N D I X

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STREPTOMYCIN RESISTANCE IN PATIENTS WITH PULMONARY TUBERCULOSIS PREVIOUSLY TREATED WITH P.A.S. ALONE

BY

F. W. A. TURNBULL,* M.B., M.R.C.P.Ed.

A. T. WALLACE, M.D., D.P.H.

SHEILA STEWART,* B.Sc.

AND

J. W. CROFTON, M.D., F.R.C.P.

(From the Department of Tuberculosis and Diseases of the Respiratory System, University of Edinburgh)

In the treatment of pulmonary tuberculosis with streptomycin alone the emergence of strains of tubercle bacilli resistant to the drug is well known. That the same thing happens when isoniazid is used alone is now established (Medical Research Council, 1952a). But the possibility of resistant strains emerging when cases of pulmonary tuberculosis are treated with sodium or calcium *para*-aminosalicylate (P.A.S.) alone has received much less attention. This is perhaps partly because of the less rapid emergence of resistant organisms when an attempt is made to induce P.A.S. resistance *in vitro* (Steenken and Wolinsky, 1950) and partly because of the greater technical difficulty of the resistance tests. Nevertheless, the emergence of P.A.S.-resistant strains in a large proportion of treated cases has been reported in the literature. Some of these reports are summarized in Table I.

The results suggest that P.A.S.-resistant strains may emerge at a later period of treatment than is usual when streptomycin or isoniazid is used alone, but that, with prolonged treatment, they are isolated from a notable proportion of patients who remain sputum-positive. It is logical to imply that if these patients are later treated

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TABLE I.—*Some Previous Reports of the Emergence of P.A.S.-resistant Tubercle Bacilli in Cases Treated with P.A.S. Alone*

Authors	Total No. of Cases	Percentage from which P.A.S.-resistant T.B. Isolated	Period of Treatment (Days)
Delaude *et al.* (1949)	5	100	157–251
Medical Research Council (1950)	37	32	<84
Carstensen (1950)	79	13	184–435
Véran *et al.* (1950)	20	75	56–182
Veterans Administration (1951)..	36	20	<62
Espersen (1951)	11	73	40–354
Tempel *et al.* (1951)	37	51	<120

with streptomycin and P.A.S. in combination P.A.S. will no longer prevent the emergence of streptomycin-resistant tubercle bacilli. This probability has often been emphasized but is still ignored by many clinicians, partly perhaps because no direct evidence has, so far as we know, been provided. In addition it has been suggested that resistance to P.A.S. is transient and, by implication, not of importance to later combined treatment (Tempel *et al.*, 1951). It is the purpose of this paper to provide evidence that P.A.S.-resistant organisms may be isolated from a high proportion of patients treated with the drug alone and whose sputum remains positive, that the resistant organisms may persist in the sputum for many months after treatment has ceased, and that streptomycin-resistant organisms may emerge rapidly if such cases are later treated with both streptomycin and P.A.S.

Material and Methods

The first group consists of nine patients with pulmonary tuberculosis who were treated with streptomycin, 1 g. daily, and P.A.S., 20 g. daily, for at least three months. (In one the daily dose of P.A.S. had to be reduced to 15 g. daily for five days a week at the end of two months, but her organisms were already streptomycin-resistant.) All had elsewhere had treatment with P.A.S. alone for periods varying from 6 weeks to 16 months, the exact daily dosage being in most cases unknown. The interval between the end of the course of P.A.S. alone and the beginning of the course of streptomycin and P.A.S. in combination varied from a few days to 39 months. Estimations of the drug sensitivity of the tubercle bacilli isolated from these patients, and the results of serial cultures and sensitivity tests while under treatment with streptomycin and P.A.S., were compared with similar results from a second group of five patients who began streptomycin and P.A.S. in the same doses about the same time, but who had not previously

TABLE II.—*Pre-treatment Temperatures and Radiographic State*

Case No.	Previous P.A.S. Alone	Pre-treatment Temperature*	Radiographic Classification, Pre-treatment	
			Cavitation†	Confluence‡
1	+	Moderate	+++	++
2	+	Afebrile	+++	++
3	+	,,	+++	++
4	+	,,	+++	+
5	+	Moderate	+++	+
6	+	Afebrile	++	+++
7	+	,,	+++	++
8	+	Moderate	+++	+++
9	+	High	+++	+++
10	0	Afebrile	++	++
11	0	Moderate	+++	+++
12	0	Afebrile	++	++
13	0	Moderate	+++	+++
14	0	Afebrile	+	+

* Graded as follows: At least 1 reading in pre-treatment week over 101° F. (38.3° C.)=High, over 99° F. (37.2° C.)=Moderate, none over 99° F.=Afebrile.

† Total diameters of cavities present 5 cm. or above=+++, 2 to 4.9 cm.=++, less than 2 cm.=+.

‡ Shadows confluent over three-quarters or more of one zone=+++, one-quarter to three-quarters of one zone=++, less than one-quarter of one zone=+.

received P.A.S. alone. All the patients were initially included in the current chemotherapeutic trials organized by the Medical Research Council (1953).

Cultures of the sputum were obtained on Löwenstein–Jensen medium before the beginning of the course of streptomycin and P.A.S. in combination. Further cultures were made at least at monthly intervals for six months. Streptomycin- and P.A.S.-sensitivity tests were carried out in liquid medium according to accepted techniques (Medical Research Council, 1948, 1950). Tubercle bacilli were regarded as drug-resistant if they were inhibited only by at least eight times the concentration which prevented growth of the sensitive control organism H37Rv; that is to say, if their "resistance ratio" was eight or above.

Comparability of the Two Groups.—The patients in the two groups were investigated with respect to factors in the pre-treatment state known to affect the emergence of resistant organisms. For this purpose we recorded the pre-treatment temperature and the extent of cavitation and confluence of shadows in the radiograph. The radiographic assessments were made by an independent panel unaware of the group to which the patient belonged, and the films were placed in previously defined categories. The results are recorded in Table II. It will be seen that the groups are roughly comparable, though in the first group gross cavitation was commoner.

Results (see Table III)

TABLE III.—*Results*

	Case No.:	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Previous P alone	Duration (months)	7	6	11	6	1½	16	4	3	9	0	0	0	0	0
	Interval before SP course (months)	4	10	4	14	39	8	16	0	0	—	—	—	—	—
SP course	S 1 g. daily (months)	3	4	3	3	3	3	4	3	3½	3	3½	3	3½	3½
	S 1 g. thrice weekly (months) ..	½	2	3	0	3	3	0	3	2½	3	2½	3	2½	1
P-resistance	Preceding SP course	>256	64	16	16	>256	64	>256	>32	4	½*	½	2	1	1†
	Highest reached ..	>256	64	>512	>256	>256	64	>1,028	>256	4	4	1	2	1	1
	Time after starting SP (months) ..	Initial	Initial	6	6	Initial	Initial	5	6	Initial	3	Initial	Initial	Initial	Initial
S-resistance	Height of 1st resistant culture ..	32	8	256	128	32	>32	8	8	None	None	8	None	None	None
	Time after starting SP (months) ..	2	2	3	2	2	2	4	5	—	—	5	—	—	—
	Highest reached ..	256	128	512	256	32	>256	32	8	1	1	8	1	4	1
	Time after starting SP (months) ..	5	3	6	6	2	3	4‡	5	Initial	3	5	1	6	Initial
Remarks											Sputum-negative after 90 days		Sputum-negative after 44 days		Sputum-negative after 31 days

P=P.A.S. S=Streptomycin. Times are given in months. Resistance figures are resistance ratios (see text).
* No pre-treatment sensitivity test; figure for first subsequent culture.
† Pre-treatment sensitivity on solid medium only.
‡ Two days after first resistant culture.

P.A.S. Resistance

It will be seen that in eight out of the nine patients who had previously been treated with P.A.S. alone tubercle bacilli isolated before beginning the combined streptomycin–P.A.S. course were P.A.S.-resistant, the resistance ratio varying from 16 to greater than 256 in different cases. In some cases there was a further substantial rise in the resistance ratio while on combined treatment. In the one patient, Case 9, whose bacilli were P.A.S.-sensitive before treatment, no P.A.S.-resistant organisms were subsequently isolated.

In three out of five of the patients who had not received previous P.A.S., bacilli isolated before the start of combined treatment were P.A.S.-sensitive. In the other two cases, for technical reasons, no pre-treatment culture was available for testing, but subsequent cultures were P.A.S.-sensitive. In none of these patients were P.A.S.-resistant bacilli isolated up to the end of the six months' observation period, though in three the sputum became negative.

It is clear, therefore, that P.A.S.-resistant tubercle bacilli emerged in a high proportion of those patients who had been treated with P.A.S. alone, and that the organisms might remain resistant for up to three years and more after stopping the drug.

Streptomycin Resistance

In 13 out of the 14 patients the tubercle bacilli were streptomycin-sensitive before the start of the combined course; in the thirteenth case (No. 10) the pre-treatment culture was contaminated but the bacilli were shown to be sensitive a month later.

Of those who had received previous P.A.S. alone, the one patient (Case 9) whose organisms were P.A.S.-sensitive before starting combined streptomycin and P.A.S. continued to excrete bacilli sensitive to both drugs up to the last available test, six months after starting the combined treatment. In all the eight other patients streptomycin-resistant organisms were isolated, in the first six cases within three months of starting treatment. In Case 7 the resistance ratio was 4, the upper limit of normal, after two and three months' treatment; at four months a culture slightly but definitely resistant (resistance ratio 8) was obtained for the first time, the ratio reaching 32 a few days later. In the remaining patient (Case 8) a resistance ratio of 4 was found at three months, the culture was contaminated at four months, and the first definitely resistant strain was isolated at five months. This last patient was the only one of the group whose bacilli attained only a low degree of streptomycin-resistance (resistance ratio 8); in the other seven the organisms became moderately or highly resistant.

Of the five patients who had not had previous P.A.S. alone, the sputum became negative in three (Cases 10, 12, and 14). In each case the last positive culture, on the 90th,

44th, and 31st days of treatment, remained streptomycin-sensitive throughout the six months' observation. In only one patient (Case 11) were slightly resistant organisms, with a resistance ratio of 8, isolated after five months' treatment.

Streptomycin-resistant tubercle bacilli, therefore, emerged in all of eight patients who had been previously treated with P.A.S. alone and whose organisms were P.A.S.-resistant before starting combined treatment with streptomycin and P.A.S. Of the six patients whose organisms were P.A.S.-sensitive before starting combined treatment, including one who had had previous P.A.S. alone, bacilli slightly resistant to streptomycin were isolated from only one, although in three the sputum became negative during the period of observation.

Discussion

The control group of patients who had not previously received P.A.S. alone was, of course, small. It was made smaller in the later stages of the investigation by a number of patients becoming sputum-negative. But a good deal of information is elsewhere available about the proportion of cases from which streptomycin-resistant organisms are likely to be isolated if streptomycin and P.A.S. are given in the same doses as in our patients. In the earlier Medical Research Council series the proportion did not exceed 10% (Medical Research Council, 1952b). In our group treated with previous P.A.S. alone the organisms were P.A.S.-resistant before combined treatment in eight out of nine, and in all of these streptomycin-resistant bacilli were isolated when they were later treated with streptomycin and P.A.S. in combination.

The implications of these findings are obvious. P.A.S., like streptomycin and isoniazid, should never be given alone to tuberculous patients. There is evidence that the use of streptomycin prevents or diminishes the emergence of P.A.S.-resistant organisms, just as P.A.S. prevents or diminishes the emergence of streptomycin-resistant organisms (Medical Research Council, 1950 ; Veterans Administration, 1951). If a patient is known to have had a long course, perhaps more than a month, of P.A.S. alone, it should be assumed, until the results of sensitivity tests become available, that his bacilli are P.A.S.-resistant. If chemotherapy is necessary he should be treated with streptomycin and isoniazid in combination. Finally, it is clear that tests for P.A.S. sensitivity are as important to the patient as those for sensitivity to streptomycin or isoniazid.

Summary

Tubercle bacilli from eight out of nine patients who had previously received sodium *para*-aminosalicylate (P.A.S.) alone for pulmonary tuberculosis were found to be resistant to the drug. The original course of treat-

ment lasted from 6 weeks to 16 months in different cases and the interval between the finish of the course and the resistance tests varied from a few days to 39 months.

When treated with daily streptomycin and P.A.S., streptomycin-resistant tubercle bacilli emerged in all the eight patients whose bacilli were P.A.S.-resistant before the start of the combined treatment. The streptomycin-resistant bacilli were obtained in six out of the eight cases within three months on combined treatment, in the seventh after four months, and in the eighth after five months.

By contrast a slightly streptomycin-resistant organism was isolated, after five months' treatment, from only one out of six similar cases whose organisms were P.A.S.-sensitive at the start of the combined course. Five of the cases had had no previous treatment with P.A.S. alone.

It is concluded that P.A.S.-resistant tubercle bacilli are likely to emerge if patients with pulmonary tuberculosis are treated with P.A.S. alone; that the resistant organisms may persist for long periods; and that, if the patients are later treated with P.A.S. and streptomycin, P.A.S. will then no longer prevent or diminish the emergence of streptomycin-resistant bacilli.

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VARIED DEGREES OF ISONIAZID RESISTANCE WITHIN STRAINS OF TUBERCLE BACILLI FROM SPUTUM AND PULMONARY CAVITIES

SHEILA M. STEWART

VARIED DEGREES OF ISONIAZID RESISTANCE WITHIN STRAINS OF TUBERCLE BACILLI FROM SPUTUM AND PULMONARY CAVITIES[1,2]

SHEILA M. STEWART[3]

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INTRODUCTION

Pyle (1) and Mitchison (2) have shown that tubercle bacilli of varied degrees of streptomycin resistance can be present in any one sample of sputum. Variations in isoniazid resistance have been demonstrated by Tompsett (3). The present report deals with similar investigations of the isoniazid resistance of bacterial populations in sputum and cavities of patients with pulmonary tuberculosis who had been treated with isoniazid. Estimations of the percentage of the organisms resistant to the various concentrations of isoniazid were made directly on the material examined. These estimations have been termed isoniazid population studies.

METHOD

The method consisted essentially of inoculating a series of Löwenstein-Jensen plates containing varying concentrations of isoniazid with serial dilutions of the sputum concentrate. A plate which did not contain any drug was included in each series in order to give a total viable count. The numbers of organisms resistant to the various drug concentrations were thus determined. Owing to the tendency of *M. tuberculosis* to clump, the colony counts represent "viable units" rather than individual organisms.

Preparation of plates: Fourfold dilutions of isoniazid in sterile distilled water were added to Löwenstein-Jensen medium in the proportion of one part of dilution to 100 parts of medium to give final drug concentrations of 0.06 to 64.0 γ per ml. The isoniazid-egg medium mixture was then poured into 9.0 cm. Petri dishes, approximately 30 ml. being used for each plate. The plates were inspissated at temperatures between 75° and 80°C. in a hot air inspissator for half an hour on each of two successive days. Before use, the plates were dried in an incubator at 37°C. A disc of sterile filter paper was then placed in the lid of the Petri dish to prevent any water of condensation from dropping onto the surface of the medium.

Preparation of the sputum dilutions: A morning specimen of sputum, to which had been added an equal volume of 4 per cent sodium hydroxide (4) was mechanically shaken with glass beads for ten minutes at room temperature. The mixture was then kept at 37°C. for twenty to thirty minutes, depending on the nature of the sputum. It was subsequently centrifuged at 2,500 r.p.m. for thirty minutes. After the supernatant fluid had been removed and the deposit made neutral to phenol red with 8 per cent hydrochloric acid, approximately 25 ml. of sterile distilled water were added to the container and the mixture was re-centrifuged as before. The resulting concentrate was homogenized by shaking with 2 ml. of sterile distilled water in a mechanical shaker for ten minutes. A series of tenfold dilutions of the homogenate in sterile distilled water was then prepared, from which the plates were inoculated immediately.

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[1] From the Department of Tuberculosis and Diseases of the Respiratory System, University of Edinburgh, Edinburgh, Scotland.

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[3] Royal Victoria Hospital Tuberculosis Trust Research Fellow in Bacteriology.

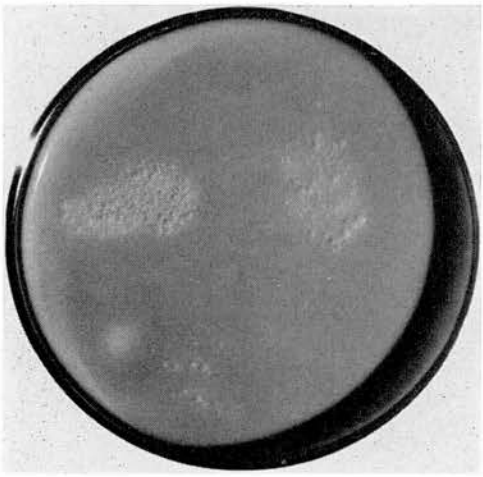

FIG. 1. Photograph showing the appearance of a plate of Löwenstein-Jensen medium after the inoculation with serial dilutions of a sputum concentrate and subsequent incubation at 37°C. for eight weeks. The highest concentration of the sputum concentrate was placed on the top left quadrant of the plate, the next on the top right, the next on the bottom left, and the lowest concentration on the bottom right quadrant.

The contents of lung cavities were treated in the same way as the sputum.

Inoculation of the plates: The plates were inoculated with Pasteur pipettes which delivered 50 drops to the ml. Two plates of each drug concentration and two control plates were inoculated from each specimen of sputum. One drop of one of four consecutive sputum dilutions was placed on the surface of each of the four quarters of every plate. The actual dilutions of the sputum concentrate used depended on the relative abundance of tubercle bacilli in the sputum. In most cases, dilutions of 1 in 10 to 1 in 10,000 were used for the control plates and the undiluted homogenate, and dilutions of 1 in 10 to 1 in 1,000 were used for the plates containing the drug. After inoculation the plates were left undisturbed at room temperature for twenty-four hours. The plates were then inverted, the filter papers removed, and paraffin wax was poured into the rim to prevent subsequent evaporation. The plates were incubated for eight weeks at 37°C. The photograph (figure 1) shows the appearance of a plate after eight weeks' incubation.

Recording of results: Counts were made from each plate at the lowest dilution of the sputum giving discrete colonies. The numbers of organisms resistant to the various drug concentrations were expressed as percentages of the total viable bacterial population.

Routine drug-susceptibility tests: Cultures were set up on Löwenstein-Jensen slopes from the neat sputum homogenate. All positive cultures were tested for isoniazid susceptibility in Löwenstein-Jensen medium at concentrations of 0.2, 1.0, 5.0, 10.0, and 50.0 γ per ml. (5).

RESULTS

Isolated Results on a Number of Patients

Studies on patients who had never received isoniazid: The results of investigations on sputum from patients who had never received isoniazid are given in table 1. It may be seen that in only one of the 8 cases (*Case 6*) was any growth found on the lowest concentration tested, i.e., 0.06 γ per ml. In that case only four colonies grew from 0.02 ml. of the undiluted sputum concentrate, i.e., only one in 4,000 of the organisms was resistant to 0.06 γ of the drug per ml. No growth

TABLE 1

THE RESULTS OF POPULATION STUDIES ON SPUTUM FROM PATIENTS WHO HAD NEVER RECEIVED ISONIAZID

Case Number	Total Viable Count per Ml. of Concentrate	Percentage of Tubercle Bacilli Resistant to Isoniazid γ *per ml.*						Isoniazid Resistance of Löwenstein-Jensen Culture*
		0.06	0.25	1.0	4.0	16.0	64.0	
1	12.5×10^5	0	0	0	0	0	0	Susceptible†
2	1.5×10^3	0	0	0	0	0	0	Susceptible
3	2.0×10^5	0	0	0	0	0	0	Susceptible
4	2.5×10^3	0	0	0	0	0	0	Susceptible
5	6.0×10^5	0	0	0	0	0	0	Susceptible
6	8.0×10^5	0.025	0	0	0	0	0	Susceptible
7	4.0×10^3	0	0	0	0	0	0	Susceptible
8	7.0×10^5	0	0	0	0	0	0	Susceptible

* By the method used in the Medical Research Council of Great Britain Trials of Isoniazid (5).

† Susceptible = fewer than 20 colonies on the slope containing 0.2 γ of isoniazid per ml. in Löwenstein-Jensen medium.

occurred at the higher concentrations. In all these cases the routine test showed the organisms to be susceptible to isoniazid concentrations of 0.2 γ per ml., the lowest concentration tested.

Studies on patients who had received isoniazid therapy and whose organisms were known to be resistant to the drug by the routine test: The results of the population studies on the sputum of 7 patients whose organisms were known to be resistant to isoniazid are shown in table 2. It will be noticed that in only one case (*Case 9*) were all of the organisms resistant to the highest concentration allowing growth. In many cases less than 1 per cent was resistant to the higher concentrations of the drug.

In the one case (*Case 9*) in which all of the tubercle bacilli appeared to be of the same degree of resistance, the organisms in the sputum were known to have been resistant to isoniazid for 516 days before the population study was carried out. The patient had not received any isoniazid for 471 days and, during this time, cultures from the sputum had shown a reduction in resistance from isoniazid concentrations of 50.0 γ per ml. to concentrations of 0.2 to 1.0 γ per ml. This low degree of resistance was maintained for eight months before the examination of the sputum. The population study showed all of the organisms in the sputum to be resistant to isoniazid concentrations of 4.0 γ per ml.

In 2 cases (*Cases 10* and *11*) the cultures from the sputum were resistant to 10.0 and 50.0 γ per ml., respectively, at the time of the population study. In each case, at least one-third of the tubercle bacilli was shown to be resistant to isoniazid concentrations of 16.0 γ per ml. These patients had been given isoniazid for 353 days and two years, respectively, and they were both receiving the drug at the time the microbial population study was carried out.

Population studies on the sputum from the remaining 4 patients all showed a marked variation in isoniazid resistance. Two of these (*Cases 12* and *13*) had

TABLE 2

THE RESULTS OF POPULATION STUDIES ON SPUTUM FROM PATIENTS WHO HAD RECEIVED THE DRUG AND WHOSE ORGANISMS WERE KNOWN TO BE RESISTANT BY THE ROUTINE DRUG TEST

Case Number	Duration of Isoniazid Therapy	Post-Treatment Interval Before Population Study	Duration of Resistance Before Population Study	Viable Count of Concentrate per Ml.	Percentage of Organisms Resistant to Isoniazid γ *per ml.*						Routine Resistance Test*
					0.06	0.25	1.0	4.0	16.0	64.0	
	days	*days*	*days*								γ *per ml.*
9	196	471	516	5.5×10^4	100	100.00	—	100.00	0	0	1.0
10	353	0	271	3.5×10^4	—	100.00	100.0	100.00	36.00	0	10.0
11	Approximately 750	0	Not known	8.5×10^5	100	100.00	100.0	100.00	33.00	4.10	50.0
12	180	420	Not known	3.5×10^5	29	36.00	1.3	0.42	0.16	0.04	50.0
13	42	264	Not known	3.5×10^5	86	57.00	11.0	1.40	0	0	5.0
14	328†	0	350	5.5×10^4	100	60.00	8.0	0.13	0	0	1.0
15	144	0	82	2.0×10^6	50	0.75	0.4	0.50	0.75	0.37	50.0

* By the method used in the Medical Research Council of Great Britain Trials of Isoniazid (5); cultures tested on Löwenstein-Jensen medium containing 0.2, 1.0, 5.0, 10.0, and 50.0 γ of isoniazid per ml.

† This patient received two courses of isoniazid of 168 and 160 days' duration, respectively, with a break of 63 days between.

received no isoniazid for 264 and 420 days, respectively. Frequent resistance tests had not been carried out on cultures from the sputum of these patients. In the third patient (*Case 14*) the organisms from the sputum had shown a reduction in degree of isoniazid resistance during the period in which the patient was not receiving the drug; during a second course of isoniazid the results on the sputum cultures varied considerably. The fourth patient (*Case 15*) had received continuous isoniazid therapy combined with oxytetracycline, 2 gm. daily, for 144 days before the population study was done. Sputum cultures before the population study had shown a low degree of resistance, but on later readings an increase was found.

The results of the routine resistance tests agreed well with the results of the population studies.

Weekly Investigations on Two Patients

It is known from the weekly routine testing of the isoniazid resistance of tubercle bacilli isolated from patients who received the drug alone that in most cases a high degree of resistance develops rapidly after the first appearance of resistant organisms (6). In an attempt to determine whether, with this rapid development of resistance, all of the organisms were affected simultaneously or whether only a few became highly resistant, weekly population studies were carried out on 2 patients.

By the time this work was begun it was already known that bacterial resistance

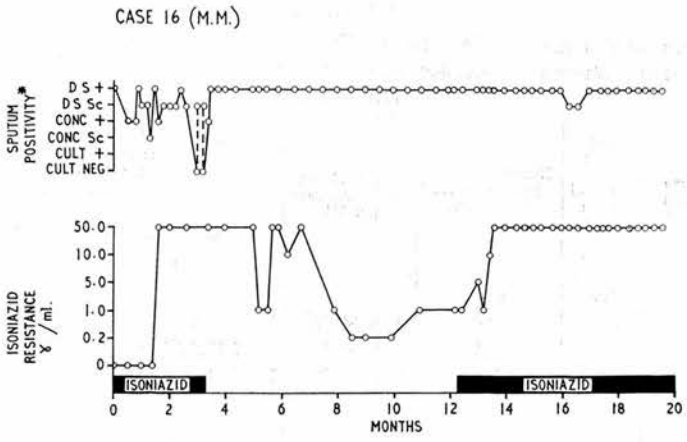

FIG. 2. Chart showing the approximate numbers of tubercle bacilli in the sputum and their isoniazid resistance during the first and second courses of isoniazid treatment of a patient (*Case 16*) on whom weekly population studies were made during the second course of isoniazid.

* DS + = Direct smear and culture positive for tubercle bacilli
DS Sc. = Direct smear with scanty number of bacilli (positive after more than five minutes' search) and culture positive
Conc. + = Concentrate film and culture positive
Conc. Sc. = Concentrate smear with scanty number of bacilli (positive after more than five minutes' search) and culture positive
Cult. + = Direct and concentrate films negative; culture positive
Cult. neg. = Direct and concentrate films and culture negative
Dotted lines indicate a positive smear with negative culture

to the drug would develop in a large proportion of patients treated with isoniazid alone (7–9). It was therefore not the usual practice to use such treatment if the patient's organisms were still susceptible to the drug. Nevertheless, there were 2 patients in the present series who had been treated with isoniazid alone in the original Medical Research Council of Great Britain Trials of Isoniazid and whose organisms were drug resistant. In neither case was any definitive therapy possible. Treatment had been stopped after the organisms had been found to be resistant to isoniazid concentrations of 50.0 γ per ml. by the routine test. During the subsequent months the tubercle bacilli from the sputum of both patients showed a marked decrease in isoniazid resistance. By the seventh month after stopping treatment in one case (*Case 16*) and the ninth month in the other (*Case 17*), the organisms were resistant only to considerably lower concentrations of isoniazid, i.e., 0.2 to 1.0 γ per ml. It was therefore decided to re-treat these patients with isoniazid alone in a dose of 100 mg. twice daily and to follow the changes that occurred in the resistance of their tubercle bacilli.

Case 16: The approximate number of tubercle bacilli in the sputum of the first patient and the results of the routine isoniazid-susceptibility tests are shown in figure 2. It will be seen that during the first course of isoniazid therapy there was some reduction in the population of tubercle bacilli in the sputum. Six weeks

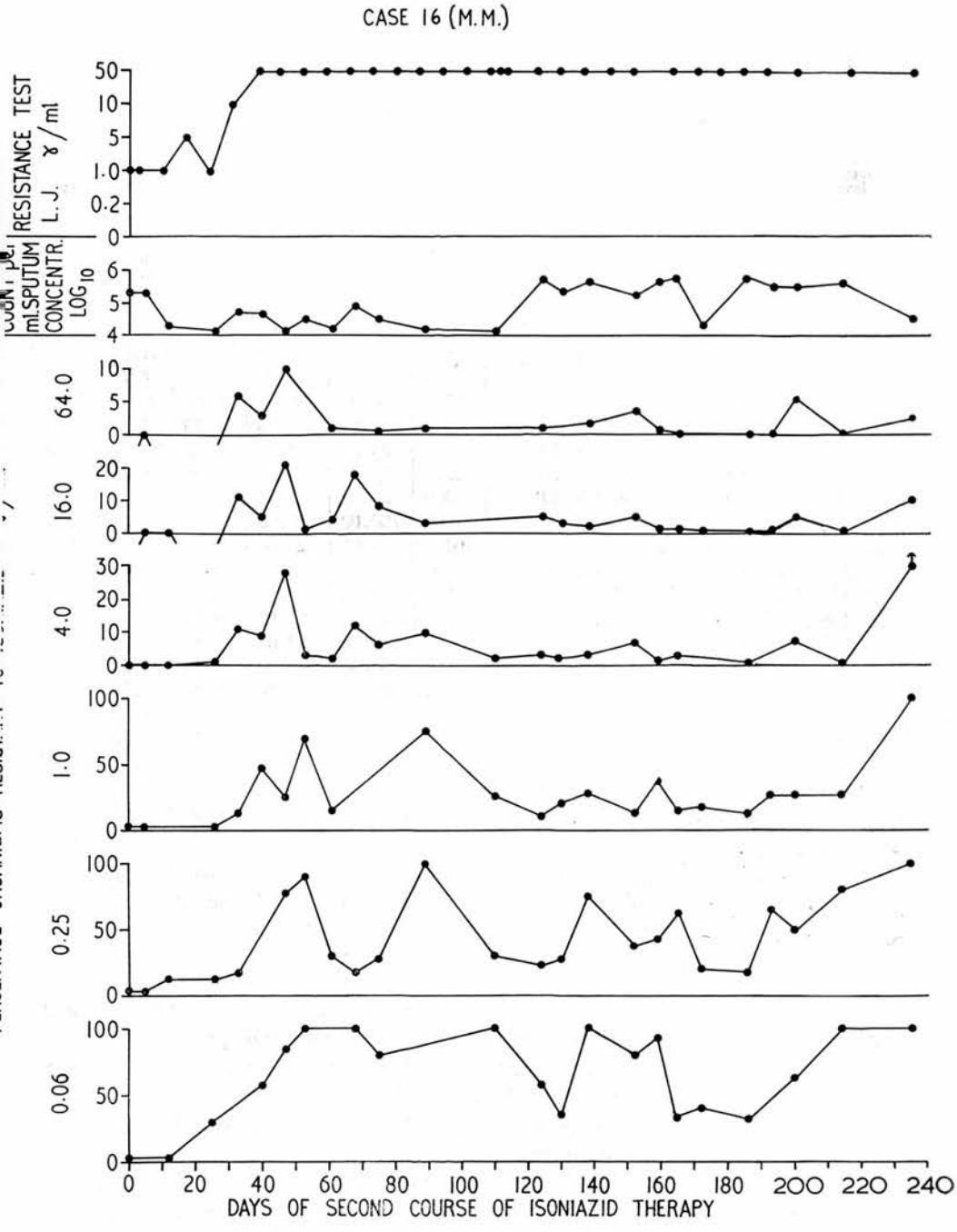

FIG. 3. Chart showing the results of the weekly population studies on the sputum during the second course of isoniazid treatment, together with the results of the routine resistance tests over this period (*Case 16*).

TABLE 3

THE RESULTS OF THE WEEKLY POPULATION STUDIES OF THE SPUTUM DURING THE SECOND COURSE OF ISONIAZID TREATMENT, TOGETHER WITH THE RESULTS OF THE ROUTINE RESISTANCE TESTS (*Case 16*)

Days After Starting Re-Treatment with Isoniazid	Isoniazid Resistance of Löwenstein-Jensen Culture*	Total Viable Count per Ml. of Sputum Concentrate	Percentage of Organisms Resistant to Isoniazid γ *per ml.*					
			0.06	0.25	1.0	4.0	16.0	64.0
	γ *per ml.*							
0	1.0	2.9×10^5	3.8	0.38	0.07	0.02	0	0
5	1.0	3.0×10^5	—	0.67	0.50	0.07	0.05	0.01
12	1.0	2.5×10^4	4.8	12.00	—	0.20	0.20	0
26	5.0	1.5×10^4	24.0	12.00	1.70	1.40	0	0
33	1.0	7.5×10^4	—	17.00	13.00	11.00	11.00	6.00
40	10.0	7.5×10^4	57.0	—	46.00	9.30	4.70	3.10
47	50.0	1.8×10^4	86.0	78.00	25.00	28.00	21.00	10.00
53	50.0	5.0×10^4	100.0	90.00	70.00	3.00	1.00	0
61	50.0	2.0×10^4	—	30.00	15.00	2.00	4.00	1.00
68	50.0	9.0×10^4	100.0	17.00	—	12.00	18.00	0
75	50.0	5.0×10^4	80.0	28.00	—	6.00	8.50	0.45
89	50.0	2.0×10^4	—	100.00	75.00	10.00	2.50	1.00
110	50.0	0.7×10^4	100.0	30.00	23.00	2.00	—	11.00
124	50.0	7.0×10^5	57.0	23.00	8.00	3.30	5.00	1.00
130	50.0	3.3×10^5	35.0	28.00	17.00	2.00	2.00	0
138	50.0	6.0×10^5	100.0	75.00	29.00	2.50	2.50	1.80
152	50.0	2.0×10^5	80.0	37.50	12.50	7.50	5.70	3.70
159	50.0	6.0×10^5	92.0	42.00	38.00	1.70	1.50	0.33
165	50.0	7.5×10^5	28.0	64.00	15.00	2.70	1.30	0.02
172	50.0	2.5×10^4	40.0	20.00	18.00	—	1.00	0
186	50.0	7.0×10^5	27.0	19.00	12.00	0.30	0.60	0.01
193	50.0	2.5×10^5	—	64.00	28.00	—	1.10	0.16
200	50.0	2.7×10^5	63.0	50.00	27.00	7.30	5.50	5.50
214	50.0	5.5×10^5	100.0	82.00	27.00	0.55	0.10	0.01
235	50.0	5.0×10^4	100.0	100.00	100.00	75.00	10.00	2.80

* By the method used in the Medical Research Council of Great Britain Trials of Isoniazid (5); cultures tested on Löwenstein-Jensen medium containing 0.2, 1.0, 5.0, 10.0, and 50.0 γ of isoniazid per ml.

after the beginning of isoniazid therapy, the patient's strain of tubercle bacilli was found to be resistant to isoniazid concentrations of 50.0 γ per ml. A disappearance of the more highly drug-resistant bacilli from the sputum occurred in the subsequent five months but, with a second course of isoniazid, there was no effect on the discharge of tubercle bacilli in the sputum. Moreover, by the sixth week of treatment, tubercle bacilli resistant to isoniazid concentrations of 50.0 γ per ml. were again present in the sputum.

The results of the weekly microbial population studies of this patient's sputum are shown in figure 3, along with the results of the routine drug-resistance tests over this period. The actual percentages of the organisms resistant to the various concentrations of isoniazid are shown in table 3. At the beginning of re-treatment, only 3.8 per cent of the tubercle bacilli were resistant to 0.06 γ of isoni-

azid per ml., and less than 0.1 per cent to concentrations of 1.0 γ per ml. or higher. By the fifty-third day of re-treatment, 100 per cent of the organisms were resistant to 0.06 γ per ml. About this time an increase also occurred in the number of organisms resistant to the higher concentrations, but at no time were 100 per cent of this patient's strain resistant to isoniazid concentrations of 4.0 γ per ml. or higher. Indeed, the percentage of the population which was resistant to 16.0 γ per ml. was never greater than 21.0 and the percentage resistant to 64.0 γ per ml. was never greater than 11.0 in spite of continuous treatment for 235 days.

Examination of 17 individual lesions obtained at post-mortem examination of this patient twenty-one weeks after the end of the second course of treatment by methods described elsewhere (10) showed a marked variation in the degrees of resistance of the tubercle bacilli cultured from the various sites.

Case 17: In figure 4 may be seen the approximate numbers of tubercle bacilli in the sputum and the resistance studies made of the strain from the second patient who was studied at weekly intervals. There was some reduction in the number of tubercle bacilli in the sputum during the first course of isoniazid until resistant organisms appeared in approximately the sixth week. A more pronounced reduction in the number of tubercle bacilli discharged in the sputum occurred during a short course of streptomycin and sodium *p*-aminosalicylate

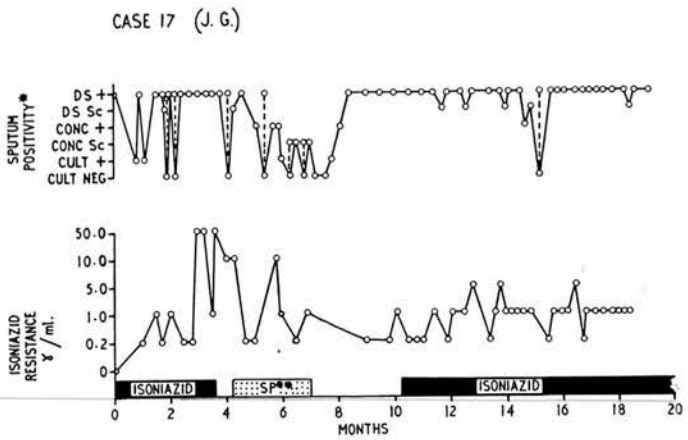

FIG. 4. Chart showing the sputum positivity and the isoniazid resistance of the tubercle bacilli during the first and second courses of isoniazid treatment of a patient (*Case 17*) on whom weekly population studies were carried out during the second course of isoniazid.

* DS + = Direct smear and culture positive
DS Sc. = Direct smear with scanty number of bacilli (positive after more than five minutes' search) and culture positive
Conc. + = Concentrate film and culture positive
Conc. Sc. = Concentrate film with scanty number of bacilli (positive after more than five minutes' search) and culture positive
Cult. + = Direct and concentrate films negative; culture positive
Cult. neg. = Direct and concentrate films and culture negative.
Dotted lines indicate a positive smear with a negative culture
** SP = Streptomycin, 1 gm. daily, with PAS, 20 gm. daily.

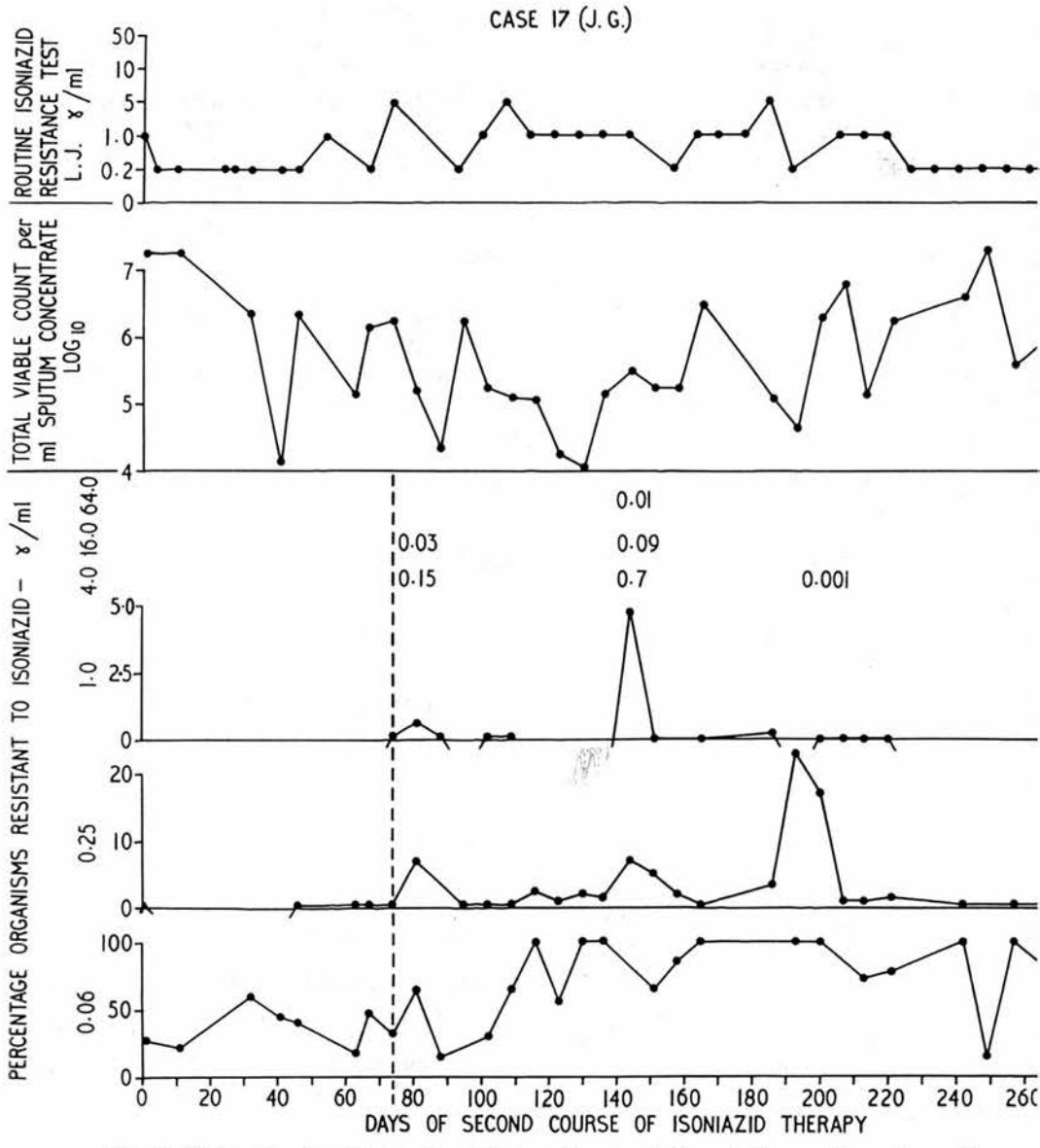

FIG. 5. Chart showing the results of the weekly population studies on the sputum of a patient (*Case 17*) during the second course of isoniazid treatment. On specimens to the left of the vertical dotted line the undiluted sputum concentrate was not inoculated onto any of the plates.

(PAS), to which drugs the patient's strain was susceptible. At this time the prognosis in this case was considered hopeless. Accordingly, streptomycin-PAS treatment was stopped after three months and the number of tubercle bacilli discharged in the sputum showed a considerable increase. The second course of isoniazid had no effect on the number of bacilli in the sputum and had only slight

effect on the level of isoniazid resistance of the strain. At no time were bacilli resistant to isoniazid concentrations of 50.0 γ per ml. observed in contrast to the situation during the first course of isoniazid therapy.

The results of the weekly population studies on the sputum from this patient may be seen in figure 5, and the detailed results are presented in table 4. It will be noticed that only 25 per cent of the organisms were resistant to isoniazid

TABLE 4

THE RESULTS OF THE WEEKLY POPULATION STUDIES ON THE SPUTUM DURING THE SECOND COURSE OF ISONIAZID TREATMENT (*Case 17*)

(On specimens above the dotted line the undiluted sputum concentrate was not inoculated onto any of the plates)

Days After Starting Re-Treatment with Isoniazid	Isoniazid Resistance of Löwenstein-Jensen Culture*	Total Viable Count per Ml. of Sputum Concentrate	Percentage of Organisms Resistant to Isoniazid γ *per ml.*					
			0.06	0.25	1.0	4.0	16.0	64.0
	γ *per ml.*							
0	0.2	3.5×10^7	25	0.06	0	0	0	0
11	0.2	2.7×10^7	22	0	0	0	0	0
32	0.2	3.4×10^6	60	0	0	0	0	0
41	1.0	1.6×10^4	44	0	0	0	0	0
46	0.2	3.3×10^6	42	0.15	0	0	0	0
63	1.0	1.4×10^5	18	0.36	0	0	0	0
67	1.0	1.9×10^6	47	0.13	0	0	0	0
74	1.0	2.2×10^6	32	0.25	0.005	0	0	0
81	5.0	1.9×10^5	65	7.00	0.650	0.150	0.03	0
88	0.2	3.3×10^4	14	—	0.150	0	0	0
95	1.0	2.3×10^6	—	0.74	0	0	0	0
102	5.0	2.5×10^5	30	0.35	0.090	0	0	0
109	1.0	1.5×10^5	65	0.70	0.170	0	0	0
116	1.0	0.7×10^5	100	2.60	0	0	0	0
123	1.0	2.8×10^4	56	0.90	0	0	0	0
130	1.0	0.5×10^4	100	2.00	0	0	0	0
136	1.0	1.5×10^5	100	1.60	0	0	0	0
144	1.0	5.0×10^5	—	7.00	4.600	0.700	0.09	0.01
151	0.2	2.5×10^5	65	5.10	0.020	0	0	0
158	1.0	2.4×10^5	84	2.10	0	0	0	0
165	1.0	5.0×10^6	100	0.55	0.004	0	0	0
186	1.0	1.0×10^5	—	3.75	0.275	0	0	0
193	1.0	6.5×10^4	100	23.00	0	0	0	0
200	5.0	3.6×10^6	100	17.00	0.050	0.001	0	0
207	0.2	8.0×10^6	—	0.80	0.001	0	0	0
213	1.0	1.3×10^5	72	0.88	0.840	0	0	0
221	1.0	2.6×10^6	77	1.50	0.030	0	0	0
242	1.0	6.3×10^6	100	0.72	0	0	0	0
249	1.0	3.1×10^7	11	0.02	0	0	0	0
257	1.0	6.0×10^5	100	0.63	0	0	0	0
271	1.0	1.2×10^6	70	0.15	0	0	0	0

* By the method used in the Medical Research Council of Great Britain Trials of Isoniazid (5); cultures tested on Löwenstein-Jensen medium containing 0.2, 1.0, 5.0, 10.0, and 50.0 γ of isoniazid per ml.

TABLE 5

THE RESULTS OF POPULATION STUDIES ON THE CONTENTS OF LUNG CAVITIES

(In one case (*Case 18*) the lung specimen was obtained at autopsy and in the other two at operation)

Case Number	Duration of Isoniazid Therapy	Post-Treatment Interval Before Population Study	Duration of Resistance Before Population Study	Viable Count of Concentrate per Ml.	Percentage of Organisms Resistant to Isoniazid γ per ml.						Resistance Test from Löwenstein-Jensen Culture*
					0.06	0.25	1.0	4.0	16.0	64.0	
	days	*days*	*days*								γ *per ml.*
18	154	209	316	6.5×10^5	—	100	100	11	1.7	0	10.0
19a	152	512	Not known	5.0×10^5	100	100	100	100	50.0	36.0	50.0
19b				3.0×10^5	100	100	100	25	13.0	6.7	50.0
20	236	8	86	5.0×10^5	100	100	18	0	0	0	2.5

* By the method used in the Medical Research Council of Great Britain Trials of Isoniazid (5); cultures tested on Löwenstein-Jensen medium containing 0.2, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, and 50.0 γ of isoniazid per ml.

concentrations of 0.06 γ per ml. before the beginning of the second course of isoniazid. It was not until approximately the one hundredth day after the reinstitution of isoniazid therapy that 100 per cent of the organisms were found to be resistant to 0.06 γ per ml. About this time there was a tendency for the results of the routine tests of this patient's organisms to rise from 0.2 γ per ml. to 1.0 γ per ml. The undiluted sputum homogenates were not used in these observations until the seventy-fourth re-treatment day. Organisms resistant to isoniazid concentrations of 0.25 γ per ml. and higher may have been present before this time but in such small numbers that they were not detected. At no time during the 270 days of re-treatment did the percentage of organisms resistant to 0.25 γ per ml. rise above 23 per cent, and this value was generally below 10 per cent. The number of organisms resistant to 1.0 γ per ml. was extremely low; at the higher concentrations of isoniazid, only a very few resistant organisms occurred, and those very infrequently.

Studies on the Contents of Lung Cavities

The results of the investigations on the contents of lung cavities from 3 patients whose organisms were known to be isoniazid resistant are shown in table 5. It will be noted that, in the cavity of the patient whose isoniazid therapy was discontinued only eight days before the population study was carried out, the resistance of the organisms was more uniform than in the other 2 patients. It should be noted, however, that this patient had received a longer course of isoniazid than had the other 2 patients. In no case were 100 per cent of the organisms resistant to the highest isoniazid concentration at which growth occurred.

DISCUSSION

Pyle (1) and Mitchison (2) have shown that organisms with varying degrees of resistance to streptomycin can be found in a single specimen of sputum from

a patient whose organisms are resistant to the drug. Tompsett (3) has demonstrated a similar variation with regard to isoniazid. His investigations were carried out on liquid medium cultures from the sputum of patients who were receiving the drug alone for the first time. He found that in many cases only a low percentage of the organisms were resistant to the higher concentrations of the drug in spite of continuous treatment for seven months. The results in the present series using undiluted sputum confirm these earlier results.

It appears that the proportion of highly resistant bacilli found in the sputum of patients with isoniazid-resistant organisms may be rather lower than is the case with streptomycin. In only one case (*Case 9*) in the present series were all of the organisms resistant to the highest concentration of the drug allowing growth (tables 2–4). In none of the contents of lung cavities was uniformity of resistance found (table 5), although the degree of variation among the individual bacilli in these lesions was less marked than among the bacilli in the sputum of other patients (table 2).

In 2 cases in the present study (*Cases 10* and *11*) all of the organisms were highly resistant to isoniazid. The patients had received the drug for 353 days and two years, respectively, and in both cases the organisms in the sputum were known to be highly resistant. It seems likely that in these cases the prolonged treatment had resulted in the development of a high degree of resistance throughout the lung.

In one case (*Case 9*) the organisms were all equally resistant, although not to a high degree. The organisms from the sputum in this patient were known to have been resistant to isoniazid concentrations of 50 γ per ml. during treatment. After the drug was stopped, however, a marked reduction occurred and, subsequently, the tubercle bacilli in the sputum were shown repeatedly to be resistant to 1.0 to 0.2 γ per ml. The uniformity of the resistance of the organisms in the sputum at the time of the population study suggests that a reduction in the number of bacilli resistant to high concentrations of the drug may have occurred throughout the lungs as has been shown to occur by the examination of individual lung lesions (10). Alternatively, the drainage from the lesions containing highly resistant bacilli may merely have become blocked.

An increase in the degree of isoniazid resistance of the organisms in the sputum was known to have occurred after the population study was done in one case (*Case 15*). The variation in the degree of isoniazid resistance of the organisms in the sputum may have been due to the fact that all of the organisms in the lung had not yet reached their maximum resistance (10).

Three patients (*Cases 14*, *16*, and *17*) received two courses of isoniazid. In each case, cultures from the sputum were highly resistant by the end of the first course of treatment, but there was a marked reduction in the degree of resistance during the time when no isoniazid was being given. In 2 cases (*Cases 14* and *16*), with the second course of isoniazid, highly resistant organisms were again isolated. In the third patient (*Case 17*), however, the organisms showed only a moderate degree of isoniazid resistance. In none of these 3 cases did all

of the organisms become resistant to the maximum concentration of isoniazid allowing growth in spite of second courses of the drug of 160, 235, and 270 days, respectively. In one of these patients (*Case 16*) the varied degree of resistance of the organisms in the sputum was probably a reflection of the marked variation of resistance of the tubercle bacilli from different lesions in the lung. The second courses of isoniazid treatment were shorter than either of the initial courses given to the 2 patients (*Cases 10* and *11*) in whom all of the organisms became highly resistant. Had the isoniazid been continued for a longer time it is possible that a greater uniformity in the degree of isoniazid resistance might have developed.

In the remaining 2 patients, resistance tests on the cultures from the sputum were obtained too infrequently to correlate them with the results of the population studies.

Canetti and Säenz (11) have shown that tubercle bacilli of varied degrees of streptomycin resistance may be isolated from different lesions within the same lung. Recent investigations in this unit have shown that a similar wide variation in isoniazid resistance can occur (10). It is therefore reasonable to suppose that sputum derived simultaneously from various sites may contain organisms of various degrees of resistance and that the proportion of the organisms resistant to the different concentrations of the drug may depend on the sources of the sputum. This may account for the marked fluctuations seen in the results shown in tables 3 and 4 and may also explain the variations which sometimes occur in a series of routine tests for isoniazid resistance on consecutive cultures from the same patient.

The clinical improvement which may occur in a patient on isoniazid therapy after the appearance of isoniazid-resistant tubercle bacilli in the sputum may be due to the greater ability of the patient's defenses to deal with the much reduced bacterial population. The results in *Cases 12*, *15*, *16*, and *17*, however, suggest that for a time the improvement may be due in part to the presence of a high percentage of isoniazid-susceptible organisms although the patient's strain appears to be resistant by a routine test.

The detailed studies on the 2 patients who were receiving a second course of isoniazid therapy gave similar results to those found by Tompsett (3) during first courses of the drug. Therefore, it may be that increases in the degree of resistance which occur with re-treatment with the drug after apparent reversion follow the same pattern as in the original development of resistance. The low percentage of organisms resistant to the higher concentrations of the drug and the similarity of these results to those found by Mitchison (2) with streptomycin-resistant organisms are consistent with the suggestion that isoniazid resistance may develop by "jump" mutations, as Bryson and Demerec (12) postulated for streptomycin.

There is a close correlation between the results of the bacterial population studies and the routine resistance tests on cultures from the same sputum (table 2). It would appear that the routine test will detect as few as 0.1 per cent of resistant organisms present in the sputum. This may be compared with a

figure of 3 per cent given by Mitchison (2) as the lowest percentage of streptomycin-resistant organisms in a liquid medium culture detectable by the routine liquid medium test. The close correlation between the highest isoniazid concentration allowing growth in the population studies and that allowing growth on the routine test would suggest that, during the normal incubation period of a primary culture of tubercle bacilli from sputum, there is no appreciable loss of isoniazid resistance.

In conclusion, these investigations show that there is usually a marked variation in the degree of isoniazid resistance in the bacilli present in the sputum of patients whose organisms have become resistant under treatment. Although this variation is also present in the organisms from individual lung lesions, it is not so marked as that in the tubercle bacilli discharged in the sputum.

SUMMARY

The percentage of tubercle bacilli in the sputum resistant to various concentrations of isoniazid was determined in 8 patients who had never received the drug. Not more than one in 4,000 of the organisms were resistant to 0.06 γ isoniazid per ml. of Löwenstein-Jensen medium.

In 5 of the 7 patients whose organisms were known to be resistant, a marked variation in the degree of resistance of individual organisms was found.

Weekly investigations were carried out during a second course of isoniazid in 2 patients whose organisms had become resistant during isoniazid therapy but had shown a reduction in the degree of isoniazid resistance following cessation of the therapy. In neither case did all of the organisms show the same degree of resistance in spite of re-treatment with isoniazid for 235 and 270 days, respectively.

Variation in the degree of isoniazid resistance among individual tubercle bacilli was present in the contents of lung cavities although the phenomenon was observed to occur to a lesser extent than in sputum.

A close correlation was found between the results of the bacterial population studies and the routine resistance tests.

SUMARIO

Grados Variables de Resistencia a la Isoniacida en Cepas de Bacilos Tuberculosos Procedentes del Esputo y de Cavernas Pulmonares

En 8 enfermos que jamás habían recibido isoniacida, se determinó en el esputo el porcentaje de microbios resistentes a varias concentraciones de la droga. No hubo más de uno en 4,000 que fuera resistente a 0.06 γ de isoniacida por ml. de medio de Löwenstein-Jensen.

En 5 de los 7 enfermos cuyos microbios se sabía que eran resistentes, observóse una notable heterogeneidad en la intensidad de la resistencia.

Durante una segunda serie de isoniacida, lleváronse a cabo investigaciones semanales en 2 enfermos cuyos gérmenes se habían vuelto resistentes durante la primera serie, pero que habían mostrado después disminución en la intensidad de su resistencia a la isoniacida. Ni en uno ni en otro caso, mostraron todos los microbios el mismo grado de resistencia a pesar de una terapéutica continua durante 235 y 270 días, respectivamente.

Notóse también heterogeneidad de la resistencia en el contenido de las cavernas pulmonares, aunque en menor grado que en el esputo.

Observóse notable correlación entre los resultados de los estudios de la población bacteriana y las pruebas habituales de la resistencia.

RESUME

Les divers degrés de la résistance a l'isoniazide dans des souches de bacilles tuberculeux extraits de crachats et de cavernes pulmonaires

Le pourcentage de germes extraits de crachats qui présentaient une résistance à diverses concentrations d'isoniazide a été déterminé chez 8 malades n'ayant jamais reçu l'antibiotique. Parmi 4.000 de ces bacilles, un seul présentait une résistance à 0,06 γ d'isoniazide par ml. du milieu de Löwenstein-Jensen.

Chez 5 des 7 patients porteurs de germes reconnus résistants, une hétérogénéité marquée dans le degré de résistance a été observée. Des investigations hebdomadaires ont été poursuivies durant une seconde cure d'isoniazide chez deux patients dont les bacilles avaient acquis la résistance au cours de la première cure, mais qui avaient ultérieurement présenté une réduction de degré de l'isoniazide-résistance. En dépit d'un traitement continu de 235 et 270 jours, respectivement, les germes ne présentaient pas tous le même degré de résistance.

L'hétérogénéité de la résistance existait dans le contenu des excavations pulmonaires, mais elle était moins marquée que dans l'expectoration.

Une étroite corrélation a été observée entre les résultats des études sur la population bactérienne et les tests de résistance classiques.

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STUDIES ON THE DISTRIBUTION OF DRUG-RESISTANT TUBERCLE BACILLI WITHIN THE LUNG

F. W. A. TURNBULL AND SHEILA M. STEWART

STUDIES ON THE DISTRIBUTION OF DRUG-RESISTANT TUBERCLE BACILLI WITHIN THE LUNG[1,2]

F. W. A. TURNBULL AND SHEILA M. STEWART[3]

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INTRODUCTION

When bacterial resistance to streptomycin or to isoniazid occurs in cases of pulmonary tuberculosis, organisms of various degrees of resistance may be isolated from a single sample of sputum (1–3). Variations may also be found in the degree of resistance of tubercle bacilli from different lesions within the same lung (3–8). The present report deals with a detailed study of the distribution of streptomycin- and isoniazid-resistant tubercle bacilli in specimens of lung tissue.

MATERIALS AND METHODS

Lung tissue was examined from 13 patients whose organisms were known to be resistant to both streptomycin and isoniazid. These patients had been treated with these drugs on regimens which would not now be used, as they are known to be ineffective in preventing the appearance of drug-resistant organisms. Isoniazid-resistance tests were carried out in all 13 cases, but streptomycin-resistance tests were only done in 8 of them. Eight patients (*Cases 1, 4, 5, 6, 7, 9, 11,* and *12*) were examined post mortem, and specimens of lung tissue were obtained from the other 5 patients (*Cases 2, 3, 8, 10,* and *13*) at operation. All cases were classified as far advanced pulmonary tuberculosis with cavitation.

Sampling of lesions: The specimens were examined within six hours of the operation or within twenty-four hours of the patient's death, except in one case (*Case 11*) in which post-mortem examination was delayed for seventy-two hours. The main sites of the disease were located by means of a recent roentgenogram and samples were taken from each of these. In addition, a number of lesions were sampled which were not visible on the roentgenogram. Precautions were taken to minimize the spread of tubercle bacilli from one site of infection to another. A small incision was made through the wall of the cavities and the contents were removed. Caseous foci were cut out and macerated with scissors.

Isolation of tubercle bacilli: The specimens were suspended in a small volume of sterile distilled water and an equal volume of 4 per cent sodium hydroxide was added. After the addition of glass beads, the mixture was shaken mechanically for ten minutes and then placed at 37°C. for twenty minutes. After centrifuging at 2,500 r.p.m. for twenty minutes, the supernatant fluid was poured off and the deposit was made neutral to phenol red with 8 per cent hydrochloric acid. Twenty-five milliliters of sterile distilled water were added and the material was centrifuged as before. The supernatant fluid was poured off. A Ziehl-Neelsen film was made from part of the deposit and the remainder was inoculated onto three Löwenstein-Jensen slopes by means of a Pasteur pipette. Cultures were incubated at 37°C. They were examined weekly and only discarded as negative after twelve weeks' incubation. Before the addition of the alkali, the aqueous suspensions of some of the specimens were injected into guinea pigs. All of the animals were killed twelve weeks after inoculation and cultures were made from the lesions.

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[1] From the Department of Tuberculosis and Diseases of the Respiratory System, University of Edinburgh, Edinburgh, Scotland.

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[3] Royal Victoria Hospital Tuberculosis Trust Research Fellow in Bacteriology.

Bacterial drug-resistance tests: In all cases, positive cultures were tested for resistance to isoniazid and in 8 cases streptomycin-resistance tests were also done. When all three cultures from a lesion were positive, the slope with the heaviest growth was used. When primary cultures were negative but guinea pig inoculation was positive, resistance tests were carried out on the tubercle bacilli isolated from the animal organs.

The streptomycin-resistance tests were done by the method recommended by the Medical Research Council of Great Britain (9), using the liquid medium of Davis and Dubos. The actual concentrations of streptomycin added to the medium were 128, 32, and 8 γ, and then, by twofold dilutions, to 0.125 γ per ml. In 2 of the cases, the organisms from the sputum were found to be susceptible by this test, but resistant when tested on Löwenstein-Jensen medium by a method already reported (10). Therefore, in these cases both liquid and solid medium drug-susceptibility tests were done on the cultures from the lung lesions. The actual concentrations of streptomycin added to the solid medium were 64.0 to 1.0 γ per ml. by twofold dilutions. The results of both methods were expressed as resistance ratios, that is, the ratio of the minimum inhibitory concentration for the test organism to the minimum inhibitory concentration for the standard H37Rv strain of *M. tuberculosis*.

The method for detecting isoniazid resistance was that recommended by the Medical Research Council of Great Britain (9), using Löwenstein-Jensen medium. The isoniazid concentrations were 50.0, 25.0, 10.0, 5.0, 2.5, 1.0, 0.5, and 0.2 γ per ml. The results were expressed as the highest concentration allowing the growth of 20 or more colonies.

Streptomycin- and isoniazid-resistance tests were also done on the last culture of tubercle bacilli isolated from the sputum before examination of the lungs. For the sputum cultures, the range of streptomycin concentrations used was 8.0 to 0.125 γ per ml. by twofold dilutions. The isoniazid resistance was only tested at 50.0, 10.0, 5.0, 1.0, and 0.2 γ per ml.

Cultures were considered to be resistant to streptomycin when a resistance ratio of 4 or more was obtained. This means that growth was inhibited by at least 1.0 γ per ml. of liquid medium, or at least 16.0 γ per ml. of solid medium. A resistance ratio of 4 by either method has been shown to be outside the normal range at the 1 per cent level (10). Clinical evidence of the significance of this degree of resistance will be published elsewhere. A growth of 20 or more colonies on the slope containing 0.2 γ of isoniazid per ml. was taken as indicating "doubtful" resistance, and a similar growth on 1.0 γ per ml. as "definite" resistance (9).

RESULTS

Streptomycin

Resistance of tubercle bacilli from the lung lesions: In table 1 may be seen the results for 8 patients of streptomycin-resistance tests in liquid medium on cultures from the lung lesions and from the last available culture of the sputum. Details are also given of the courses of chemotherapy, the times since streptomycin was last administered, and the duration of streptomycin resistance before the examination of the lung.

Owing to the experimental errors of the resistance test, cultures from the lesions were considered to show a significant variation in the degree of streptomycin resistance only if there was at least a fourfold difference between the results. This difference between the maximum and minimum degrees of resistance of the lung cultures was present in 6 cases. The resistance ratios varied from 64 to greater than 256 in one case (*Case 10*); from 1 to greater than 256 in another (*Case 11*); from 1 to 16 in a third (*Case 3*); and from 1 to 8 in a fourth (*Case 7*). In 2 cases (*Cases 1* and *4*) the readings varied from 16 to 256. In the remaining 2 cases (*Cases 5* and *6*), cultures from all the lesions had a resistance ratio of greater than 256.

TABLE 1

DISTRIBUTION OF STREPTOMYCIN RESISTANCE WITHIN THE LUNGS

Case Number	Chemotherapy		Post-Streptomycin Interval Before Examination of Lung	Duration of Streptomycin Resistance Before Examination of Lung	Streptomycin Resistance of Last Sputum Culture (Resistance Ratio†)	Type of Lesion	Total Number of Lesions	Streptomycin Resistance (Resistance Ratio†)									
	Drugs*	Months						1	2	4	8	16	32	64	128	256	>256
			months	*months*													
1	PAS	7	5	Not	16	Cavities	3	—	—	—	—	1	—	1	—	1	—
	SM-(PAS)	3		known		Caseous foci	2	—	—	—	—	—	—	1	—	1	—
	(SM_2-PAS)	8															
	1 gm. Oxyt.-INH	3															
3	PAS	7	None	Not	1‡	Cavities	1	1‡	—	—	—	—	—	—	—	—	—
	SM_3-(PAS)	1½		known		Caseous foci	6	1‡	—	1	1	3	—	—	—	—	—
	SM_2-(PAS)	3															
	SM-(PAS)	2															
	(SM_3)-INH	2															
	(SM)-INH	5															
4	PAS	4	5	24	>32	Cavities	3	—	—	—	—	1	—	1	—	1	—
	INH	3				Caseous foci	2	—	—	—	—	2	—	—	—	—	—
	SM-(PAS-INH)	1															
	SM-(PAS)	2															
	(SM-PAS-INH)	1															
	5 gm. Oxyt.-(SM)	1½															
	(SM-PAS-INH)	9															
5	SM_3-PAS	7	18	21	>16	Caseous foci	7	—	—	—	—	—	—	—	—	—	7
	2 gm. Oxyt.-INH	6															
6	PAS	4	None	6	>16	Cavities	1	—	—	—	—	—	—	—	—	—	1
	INH	6				Caseous foci	5	—	—	—	—	—	—	—	—	—	5
	2 gm. Oxyt.-SM	8															

7	PAS	10	6	>12	2‡	Right lung:										
	SM-(PAS)	7				Cavities	3	—	—	—	3	—	—	—	—	—	—
	(SM_3-PAS)	3				Caseous foci	3	—	—	2	1	—	—	—	—	—	—
	(SM-PAS)-INH	5				Left lung:											
						Cavities	2	—	1	1	—	—	—	—	—	—	—
						Caseous foci	4	1‡	1‡	1	1	—	—	—	—	—	—
10	SM-PAS	6	7	10	>16	Cavities	2	—	—	—	—	—	—	—	—	—	2
	SM_3-PAS	6				Caseous foci	4	—	—	—	—	—	—	1	—	—	3
	2 gm. Oxyt.-INH	5															
11	PAS	9	3	8	>16	Right lung:											
	INH	3				Cavities	5	1	—	—	—	1	—	—	1	—	2
	SM-(PAS)	2				Caseous foci	11	—	—	1	—	1	—	—	1	—	8
	(SM-INH)	3				Left lung:											
						Cavities	1	—	—	—	1	—	—	—	—	—	—
						Caseous foci	2	—	—	—	—	1	1	—	—	—	—

* SM—streptomycin; PAS—para-aminosalicylic acid; INH—isoniazid; Oxyt.—oxytetracycline. The dosage of streptomycin is indicated by the numeral after the letters SM, e.g., SM_2 = streptomycin twice weekly. SM alone indicates daily streptomycin.

The symbols in parentheses indicate that the patient's organisms were resistant to these drugs.

† By the method used in the Medical Research Council of Great Britain Trials of Isoniazid (9) using twofold dilutions of the drug in liquid medium of Davis and Dubos. The results are expressed as resistance ratios, that is, the ratio of the minimum inhibitory concentration for the test strain to the minimum inhibitory concentration for the standard H37Rv strain of *M. tuberculosis.*

‡ Resistant when tested on Löwenstein-Jensen medium (*see* text).

In 2 cases (*Cases 3* and *7*) the last available cultures from the sputum were drug susceptible when tested in the liquid medium, the resistance ratios being 1 and 2, respectively. Both of these cultures were resistant when tested on solid medium, a resistance ratio of 8 being obtained in each case. Furthermore, in each case, cultures from two lesions which were drug susceptible by the liquid medium test were shown to be resistant when tested on the solid medium. In *Case 5*, the culture from a third lesion, which was drug susceptible in liquid medium, did not grow on the solid medium test.

Comparison of the degree of resistance of cultures from cavities and caseous foci: It will be seen in table 1 that in 2 cases (*Cases 1* and *7*) there was no difference between the degree of streptomycin resistance of tubercle bacilli isolated from the cavities and caseous foci. In *Cases 6*, *10*, and *11*, no difference could be demonstrated since organisms from both types of lesions were isolated which were resistant to the maximum concentration tested. In the sixth case (*Case 4*) the maximum degree of resistance was present in cultures from a cavity. In the seventh case (*Case 3*) the maximum degree of resistance was present in cultures from a caseous focus. In the eighth case (*Case 5*) resistance tests were not available on cultures from the cavities.

Isoniazid

Drug resistance of tubercle bacilli from the sputum: The results of isoniazid-resistance tests on cultures from the sputum of the 13 patients, together with the duration of therapy with isoniazid alone or along with other drugs are shown in figure 1. When available, the results are given from the beginning of the first course of isoniazid to the time of examination of the lung specimens. The range of resistance of cultures from the lungs is also given.

Resistance of cultures from the lung lesions: In table 2 and figure 1 are shown the results of the resistance tests on cultures from the individual lung lesions. In each of the first 9 patients it will be seen that the degree of resistance of cultures from the lesions lay within three consecutive concentrations of isoniazid. This variation is inherent in the method used for detecting isoniazid resistance and, therefore, in these patients the resistance of the cultures from the lesions are considered to be uniform. In terms of the highest isoniazid concentrations permitting growth, the readings were 0.2 to 1.0 γ per ml. in one patient (*Case 6*), 1.0 to 2.5 γ per ml. in 3 patients (*Cases 1*, *2*, and *5*), 0.5 to 2.5 γ per ml. in a fifth patient (*Case 3*), 2.5 to 5.0 γ per ml. in a sixth (*Case 4*), and 10.0 to 50.0 γ per ml. in a seventh patient (*Case 7*). In the other 2 patients (*Cases 8* and *9*), the tubercle bacilli isolated from all of the lesions were resistant to isoniazid concentrations of 50.0 γ per ml., the highest concentration tested.

In the remaining 4 patients, the degrees of resistance within the lungs showed greater variation than could be accounted for by the method. In one patient (*Case 10*), the tubercle bacilli from one lesion were completely susceptible; in the other four lesions, the isoniazid resistance of the bacilli ranged from 5.0 to 50.0 γ of the drug per ml. Cultures from the lung of a second patient (*Case 11*) varied in isoniazid resistance from 0.2 to 25.0 γ per ml. The tubercle bacilli

TABLE 2

DISTRIBUTION OF ISONIAZID RESISTANCE WITHIN THE LUNGS

Case Number	Chemotherapy		Post-Isoniazid Interval Before Examination of Lung	Duration of Isoniazid Resistance Before Examination of Lung	Isoniazid Resistance of Last Sputum Cultures†	Type of Lesion	Total Number of Lesions	Isoniazid Resistance† *γ per ml.*								
	Drugs*	Months						0	0.2	0.5	1.0	2.5	5	10	25	50
			months	*months*	*γ per ml.*											
1	PAS	7	None	1	1.0	Cavities	3	—	—	—	3	—	—	—	—	—
	SM-(PAS)	3				Caseous foci	2	—	—	—	1	1	—	—	—	—
	(SM_2-PAS)	8														
	1 gm. Oxyt.-INH	3														
2	PAS	5	6	9	1.0	Cavities	4	—	—	—	1	3	—	—	—	—
	SM-(PAS)	4½				Caseous foci	1	—	—	—	1	—	—	—	—	—
	(SM-PAS)-INH	3														
	(SM)-INH	4														
3	PAS	7	¼	3	1.0	Cavities	1	—	—	—	—	1	—	—	—	—
	SM_3-(PAS)	1½				Caseous foci	6	—	—	1	3	2	—	—	—	—
	SM_2-(PAS)	3														
	SM-(PAS)	2														
	(SM_3)-INH	2														
	(SM)-INH	5														
4	PAS	4	4	25	5.0	Cavities	4	—	—	—	—	2	2	—	—	—
	INH	3				Caseous foci	2	—	—	—	—	2	—	—	—	—
	SM-(PAS-INH)	1														
	SM-(PAS)	2														
	(SM-PAS-INH)	1														
	5 gm. Oxyt.-(SM)	1½														
	(SM-PAS-INH)	9														
5	SM_3-PAS	7	11½	15½	5.0	Caseous foci	7	—	—	—	3	4	—	—	—	—
	2 gm. Oxyt.-INH	6														

TABLE 2—*Continued*

DISTRIBUTION OF ISONIAZID RESISTANCE WITHIN THE LUNGS

Case Number	Chemotherapy		Post-Isoniazid Interval Before Examination of Lung	Duratioo of Isoniazid Resistance Before Examination of Lung	Isoniazid Resistance of Last Sputum Cultures†	Type of Lesion	Total Number of Lesions	Isoniazid Resistance† *γ per ml.*								
	Drugs*	Months						0	0.2	0.5	1.0	2.5	5	10	25	50
			months	*months*	*γ per ml.*											
6	PAS	4	21	26	1.0	Right lung:										
	INH	6				Caseous foci	4	—	1	2	1	—	—	—	—	—
	2 gm. Oxyt.-SM	8				Left lung:										
						Cavities	1	—	—	1	—	—	—	—	—	—
						Caseous foci	1	—	—	1	—	—	—	—	—	—
7	PAS	10	7	10	50.0	Right lung:										
	SM-(PAS)	7				Cavities	3	—	—	—	—	—	—	2	1	—
	(SM_3-PAS)	3				Caseous foci	3	—	—	—	—	—	—	2	1	—
	(SM-PAS)-INH	5				Left lung:										
						Cavities	2	—	—	—	—	—	—	—	—	2
						Caseous foci	4	—	—	—	—	—	—	—	—	4
8	PAS	3½	15	Not	50.0	Cavities	3	—	—	—	—	—	—	—	—	3
	INH	2½		known		Caseous foci	7	—	—	—	—	—	—	—	—	7
	SM-INH	½														
	INH	2														
	SM_3-PAS	7½														
9	PAS	4½	2	23	50.0	Right lung:										
	SM-PAS	4				Cavities	6	—	—	—	—	—	—	—	—	6
	INH	1½				Caseous foci	5	—	—	—	—	—	—	—	—	5
	(SM_3-INH)	1				Left lung:										
	(SM_3-PAS-INH)	3				Cavities	4	—	—	—	—	—	—	—	—	4
	(SM-PAS-INH)	2				Caseous foci	1	—	—	—	—	—	—	—	—	1

10	SM-PAS SM_3-PAS 2 gm. Oxyt.-INH	6 6 5	None	1½	50.0	Cavities Caseous foci	2 4	— 1	— —	— —	— —	— —	— 1	— 1	— 1	2 —
11	PAS INH SM-(PAS) (SM-INH)	9 3 2 3	3	Not known	10.0	Right lung: Cavities Caseous foci Left lung: Cavities Caseous foci	 5 11 1 2	 — — — —	 2 1 — —	 1 1 — —	 — 1 — 1	 — 1 — 1	 — 4 1 —	 1 2 — —	 1 1 — —	 — — — —
12	INH (INH)	4 12	6	29	50.0	Right lung: Cavities Caseous foci Left lung: Cavities Caseous foci	 4 6 3 4	 — — — —	 — — — —	 — — — —	 2 — — —	 1 1 — —	 1 — 1 2	 — 1 — —	 — 1 — —	 — 3 2 2
13	SM-PAS SM_3-PAS 5 gm. Oxyt.-INH 5 gm. Oxyt.-(INH) (SM-PAS-INH)	3½ 6 6 1 4½	7	15½	5.0	Cavities Caseous foci	2 7	— —	— —	— —	— 1	— 1	2 3	— 1	— 1	— —

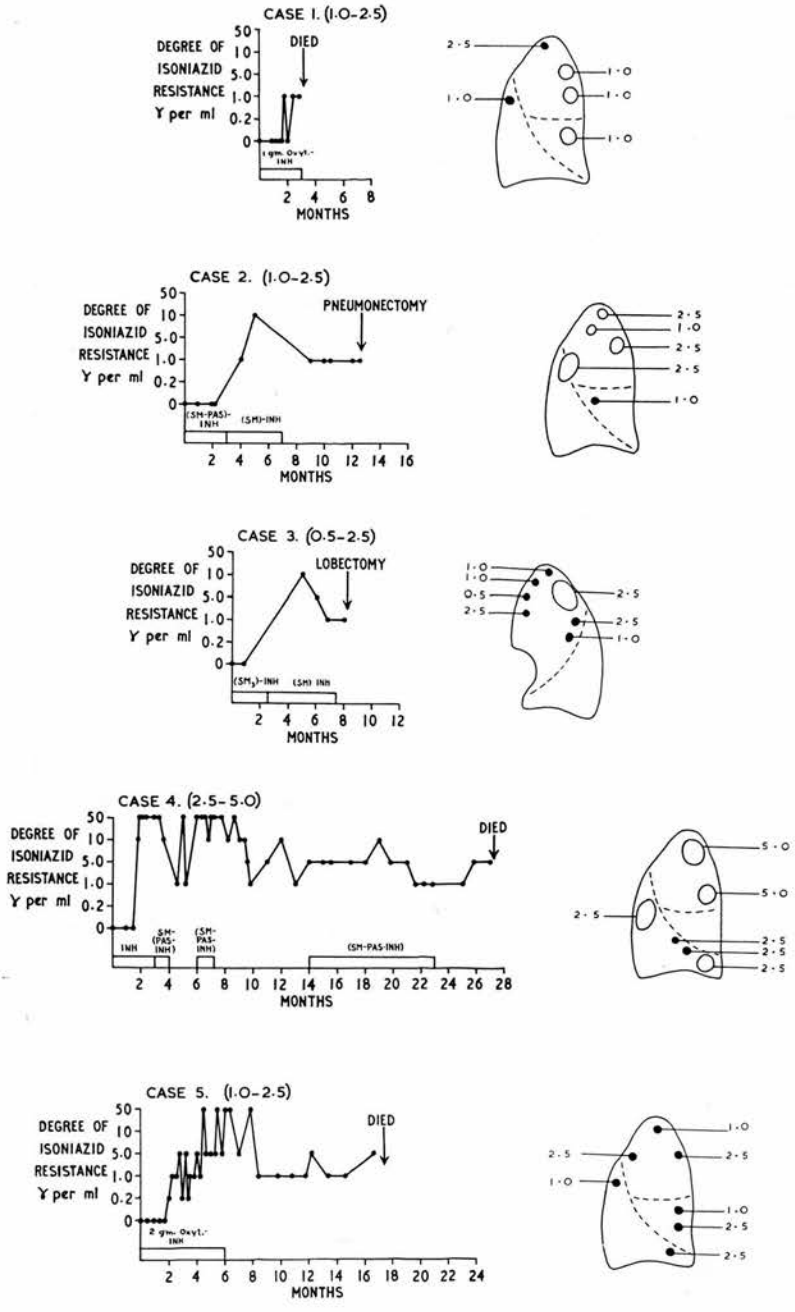
* SM—streptomycin; PAS—para-aminosalicylic acid; INH—isoniazid; Oxyt.—oxytetracycline. The dosage of streptomycin is indicated by the numeral after the letters SM.

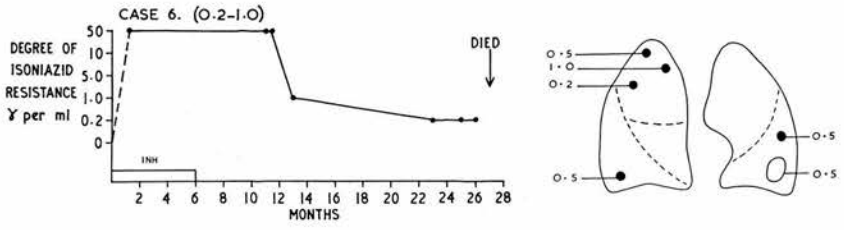
The symbols in parentheses indicate that the patient's organisms were resistant to these drugs.

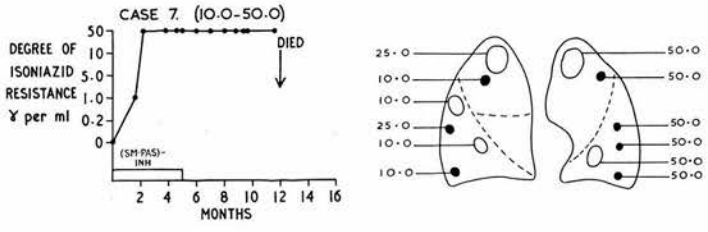
† By the method used in the Medical Research Council of Great Britain Trials of Isoniazid (9). Cultures from the sputum were tested on Löwenstein-Jensen medium containing 0.2, 1.0, 5.0, 10.0, and 50.0 γ of isoniazid per ml., and those from the lung lesions at concentrations of 0.2, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, and 50.0 γ of isoniazid per ml.

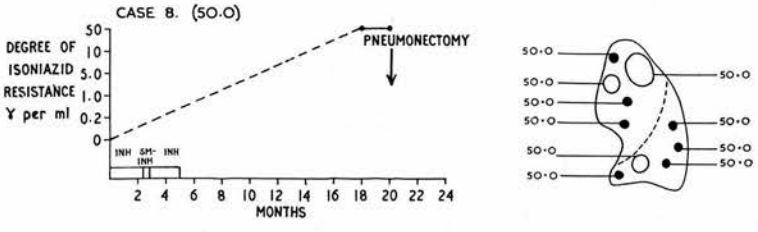
FIGURE 1

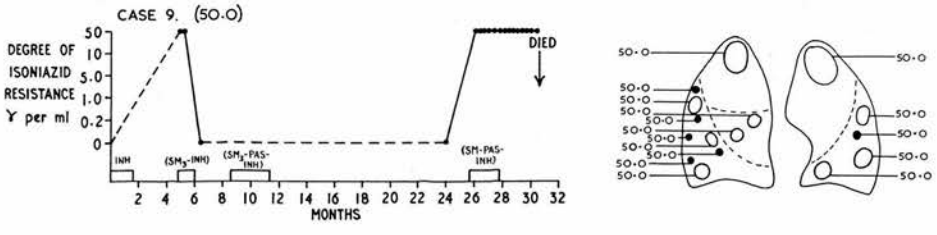
Graphs showing the result of isoniazid-resistance tests on cultures from the sputum of the 13 patients together with the duration of isoniazid therapy alone or administered along with other drugs.




CASE 6. (0.2-1.0)
DEGREE OF ISONIAZID RESISTANCE γ per ml
50
10
5.0
1.0
0.2
0
DIED
INH
2 4 6 8 10 12 14 16 18 20 22 24 26 28
MONTHS
0·5
1·0
0·2
0·5
0·5
0·5


CASE 7. (10.0-50.0)
DEGREE OF ISONIAZID RESISTANCE γ per ml
50
10
5.0
1.0
0.2
0
DIED
(SM·PAS)-INH
2 4 6 8 10 12 14 16
MONTHS
25·0
10·0
10·0
25·0
10·0
10·0
50·0
50·0
50·0
50·0
50·0
50·0


CASE 8. (50.0)
DEGREE OF ISONIAZID RESISTANCE γ per ml
50
10
5.0
1.0
0.2
0
PNEUMONECTOMY
INH SM-INH INH
2 4 6 8 10 12 14 16 18 20 22 24
MONTHS
50·0
50·0
50·0
50·0
50·0
50·0
50·0
50·0
50·0


CASE 9. (50.0)
DEGREE OF ISONIAZID RESISTANCE γ per ml
50
10
5.0
1.0
0.2
0
DIED
INH
(SM2-INH)
(SM1-PAS-INH)
(SM-PAS-INH)
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
MONTHS
50·0
50·0
50·0
50·0
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50·0
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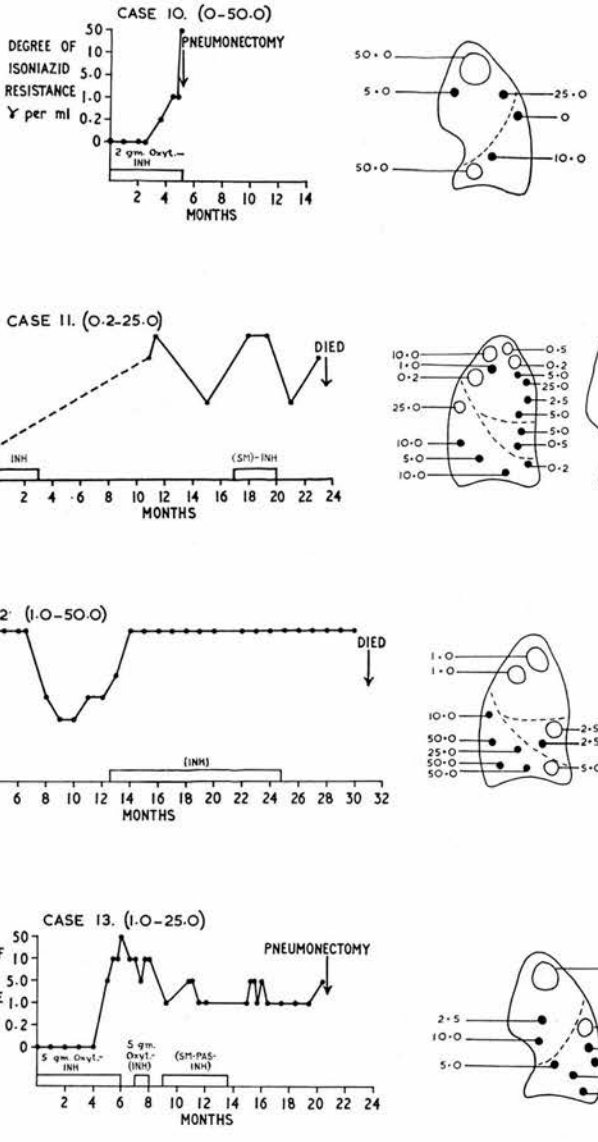
50·0
50·0
50·0
50·0
50·0
50·0
50·0



The range of resistance of cultures from the lungs is given in parentheses after the case number, and the distribution of resistance is shown in the diagrams at the end of each graph. The blocks indicate duration of therapy. The degree of isoniazid resistance is given in γ of isoniazid per ml. of Löwenstein-Jensen medium, the concentrations tested being 50.0, 10.0, 5.0, 1.0, and 0.2 γ of isoniazid per ml. The method used was that of the Medical Research Council of Great Britain Trials of Isoniazid (9). The figure 0 indicates a growth of less than 20 colonies at 0.2 γ per ml., that is, complete susceptibility.

SM—streptomycin; PAS—para-aminosalicylic acid; INH—isoniazid; Oxyt.—oxytetracycline. The dosage of streptomycin is indicated by the numeral after the letters SM, e.g., SM_2 = streptomycin twice weekly. SM alone indicates daily streptomycin.

from a third patient (*Case 12*) were resistant to isoniazid concentrations of 1.0 to 50.0 γ per ml., and in a fourth patient (*Case 13*) the degrees of resistance varied from 1.0 to 25.0 γ of isoniazid per ml.

Allowing for experimental error, the degree of resistance of the last culture of the sputum which was tested was in agreement with the maximum degree of resistance of the cultures from the lung in each of the 13 patients.

Comparison of the degree of resistance of tubercle bacilli obtained from cavities and from caseous foci: A comparison of the degree of isoniazid resistance of tubercle bacilli from cavities and from caseous foci is shown in table 2. In the 9 cases in which a uniform degree of resistance was present in the lung lesions, it was evident that no difference could occur between the degree of resistance in the two types of lesion. In 2 (*Cases 11* and *12*) of the 4 patients showing a varied degree of resistance, the maximum degree of resistance was found in both the cavities and the caseous foci. In the third patient (*Case 10*) the maximum degree of resistance was found in the cavities; and in the fourth (*Case 13*), in the caseous foci.

Comparison of the degree of streptomycin and isoniazid resistance within the same lesion: In only 2 of the patients who showed a varied degree of isoniazid resistance (*Cases 10* and *11*) were streptomycin-resistance tests also done on tubercle bacilli from the same lesions. In neither case was the maximum degree of resistance to one drug found only in cultures from lesions containing organisms with the maximum degree of resistance to the other drug.

DISCUSSION

Säenz and Canetti (4, 5) have shown that tubercle bacilli with various degrees of streptomycin resistance can be found within the same lung. Other workers (6–8) have confirmed these findings, and similar results have been found in the present series of cases.

Canetti and Säenz (5) reported that a high degree of streptomycin resistance is more often found in cavities than in caseous foci. It has not been possible to confirm this in the present study. In the Canetti-Säenz studies, the incidence of highly resistant organisms in the two types of lesion was expressed as the percentage of the total number of each lesion examined. Owing to the wide variety of chemotherapeutic regimens used in the present study, it was of little use to summate the results from all cases. Consequently, a comparison was made of the degree of streptomycin resistance of tubercle bacilli isolated from the two types of lesion in the individual lungs. In one of the 7 cases in which both cavities and caseous foci were examined, the tubercle bacilli with the maximum degree of streptomycin resistance were isolated from a cavity, but in a second case the maximum degree of resistance was found in a caseous focus. In the remaining 5 cases there was no difference in the degree of resistance found in the two types of lesion.

The finding of higher degrees of streptomycin resistance in cavities may depend on the duration of streptomycin therapy. Of the eleven cases reported by Canetti and Säenz (5) in which the duration of streptomycin therapy is

given, all but two had less than four months' treatment. In these two cases the degree of resistance of the organisms in the cavities was not higher than that of those in the caseous foci. In the present series, all of the patients had received a minimum of five months' treatment and the majority had received treatment for a considerably longer period.

It has been reported that, within the same lung, some lesions may contain tubercle bacilli susceptible to streptomycin although tubercle bacilli from other lesions may be resistant to this drug (5–7). In the lungs from six patients, all of whom had been treated with streptomycin for at least one hundred and twenty days, Fisher (11) did not find any foci containing streptomycin-susceptible organisms.

In the present series, tubercle bacilli susceptible to streptomycin by the liquid medium test were isolated from six lesions (*Cases 3*, *7*, and *11*). The cultures of bacilli from four lesions (*Cases 3* and *7*) were shown to be resistant when tested on solid medium. The remaining two cultures were not tested by this method and, therefore, it is not certain whether they were actually drug susceptible.

Organisms with various degrees of isoniazid resistance may be isolated from several lesions within the same lung (3). Such variation in the degree of isoniazid resistance was found in 4 of the 13 cases in the present series. In the remaining 9 cases, no variation in the degree of resistance was found.

There are a number of factors that may influence the uniformity of isoniazid resistance in the various lesions which merit discussion.

It has been shown that tubercle bacilli of various degrees of resistance may be present in the sputum during the development of isoniazid resistance (12). At this stage it is unlikely that the organisms within the various lung lesions would have reached a uniform degree of resistance. This is seen in *Case 10*. The marked variation in the degree of resistance present in this case may be due to one or more of the following factors:

The duration of isoniazid therapy after the emergence of resistant tubercle bacilli might influence the degree of variation of resistance within the lung. With prolonged isoniazid therapy, a uniformly high degree of resistance in the lung lesions might occur. With shorter courses of the drug, variation in the degrees of resistance might be more likely.

Different concentrations of isoniazid in lesions might result in the production of organisms of various degrees of resistance. Manthei and associates (13) have shown that different concentrations of isoniazid may be found in lesions within the same lung. In one experiment, Singh and Mitchison (14) found that, when organisms were exposed to varied concentrations of isoniazid *in vitro*, the higher the concentration of the drug, the greater the degree of resistance of the organisms which survived. These findings may explain the varied degrees of isoniazid resistance within the lung.

Schaeffer (15) has shown that isoniazid has no effect on tubercle bacilli which are not multiplying. Such dormant organisms may remain viable for a considerable period in contact with the drug and on transference to a culture medium might prove to be fully susceptible. This could explain the presence of susceptible tubercle bacilli in some lesions in spite of the existence of resistant organisms in others.

In 4 of the present cases, the tubercle bacilli in the sputum showed a constant high degree of resistance even for some months after isoniazid therapy had been stopped. In 3 of the patients (*Cases 7*, *8*, and *9*), a high degree of resistance was found in all of the lung lesions. In the fourth patient (*Case 12*), marked variation in the degree of resistance was present. There is no obvious explanation for the inconsistency of these results. It might have been thought that the duration of isoniazid therapy after the development of resistance or the time since the drug was last given would influence the degree of uniformity of resistance in the tubercle bacilli in the lung. No confirmation of this supposition has been found in the particular cases studied from this standpoint.

In 5 other cases (*Cases 2*, *3*, *4*, *5*, and *6*) sputum cultures, although at some time highly resistant, subsequently showed a constant low degree of resistance. In each of these cases cultures from all of the lung lesions showed a uniformly low degree of resistance. In another case (*Case 11*) examination of cultures of the sputum had shown that reversion to a lower degree of resistance was taking place shortly after the second course of isoniazid had been stopped. A wide variation was found in the degree of resistance of the lung cultures. One or more of the following factors might explain these results:

> If resistance to isoniazid develops by an adaptive mechanism, it might be expected that the whole bacterial population would subsequently become less resistant when no longer in contact with the drug.
>
> Mitchison has suggested that reversion of streptomycin resistance may be due to the replacement of resistant organisms by more rapidly growing susceptible ones (16). It has been shown that, within an individual lesion, organisms of low degrees of isoniazid resistance can be present although by the routine susceptibility test the culture is highly resistant (12). If organisms of a low degree of resistance grow more rapidly than highly resistant strains, the more resistant bacilli may ultimately be replaced by the less resistant ones after the patient has stopped isoniazid therapy. When completely susceptible organisms remain at the end of treatment, reversion to complete susceptibility could occur.

It is difficult to explain the results in 2 of the cases (*Cases 1* and *13*). In *Case 1*, isoniazid resistance was of recent onset and there was uniformity in the degree of resistance among the bacilli from the separate lung lesions. This is in contrast to the findings in *Case 10* in which, although drug-resistant tubercle bacilli had likewise appeared only recently, the organisms from the lung lesions showed marked variations in the degree of isoniazid resistance. In *Case 13* there was evidence of a lowering to a constant low level of the degree of isoniazid resistance of the bacilli in the sputum. Nevertheless, highly resistant organisms were isolated from some of the lung lesions. Presumably the lesions which contained these organisms were not contributing to the sputum.

In the 4 cases in which there was a variation in the degree of resistance of the organisms in the lung lesions, there was no evidence that the degree of resistance was dependent upon the nature of the lesion. Although in one case

(*Case 10*) the maximum degree of resistance was found in the cavities, in one of the lungs from another patient (*Case 13*) the maximum degree was found among tubercle bacilli from the caseous foci.

In conclusion, it is evident that many factors might have an influence on the distribution of drug-resistant bacilli within the lung, but much further work will have to be done before the exact part each plays can be elucidated.

SUMMARY

An examination was made of the distribution of streptomycin- and isoniazid-resistant tubercle bacilli in specimens of lung tissue from 13 patients.

Marked variation in the degree of resistance of tubercle bacilli from the lesions within the same lung was found in 6 of the 8 cases examined for streptomycin resistance, and in 4 of the 13 examined for isoniazid resistance.

There was no apparent difference in the degree of streptomycin or isoniazid resistance of tubercle bacilli isolated from cavities and caseous foci.

SUMARIO

Estudios de la Distribución de Bacilos Tuberculosos Farmacorresistentes Dentro del Pulmón

En los ejemplares de tejido pulmonar procedente de 13 enfermos, hízose un estudio de los bacilos tuberculosos estreptomicino- e isoniacido-resistentes. En 6 de los 8 enfermos estudiados en cuanto a resistencia a la estreptomicina y en 4 de los 13 estudiados en cuanto a resistencia a la isoniacida, descubrióse notable variación en la intensidad de la resistencia de cultivos procedentes de lesiones del mismo pulmón.

No hubo diferencia aparente en la intensidad de la resistencia a la estreptomicina y la isoniacida en los microbios aislados de cavernas y focos caseosos.

Discútense los factores que pueden afectar la uniformidad de la resistencia a la isoniacida en el pulmón.

RESUME

Recherches sur la répartition dans le poumon des bacilles antibiotiques-résistants

Il a été procédé à l'examen de la répartition des bacilles streptomycino et isoniazide-résistants dans des pièces de tissu pulmonaire provenant de 13 patients. Des différences marquées dans le degré de résistance de colonies provenant de lésions situées dans un même poumon ont été découvertes chez 6 des 8 malades étudiés pour rechercher la streptomycino-résistance et chez 4 des 13 malades étudiés pour l'isoniazide-résistance.

Il n'existait aucune différence apparente dans le degré de la streptomycino ou isoniazide-résistance des germes isolés de lésions excavées ou de foyers caséeux.

Les facteurs susceptibles d'influencer l'uniformité de l'isoniazide-résistance sont étudiés.

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THE DETECTION OF STREPTOMYCIN RESISTANCE IN TUBERCLE BACILLI

BY

SHEILA M. STEWART

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TAVISTOCK SQUARE, W.C.1

THE DETECTION OF STREPTOMYCIN RESISTANCE IN TUBERCLE BACILLI

BY

SHEILA M. STEWART*

From the Department of Tuberculosis and Diseases of the Respiratory System, University of Edinburgh

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It has been shown (Dye, Lynch, and Brees, 1953; Medical Research Council, 1953a and b; Pitts, Tempel, Miller, Sands, Fitzpatrick, and Weiser, 1953) that the treatment of tuberculosis with two drugs simultaneously markedly reduces the incidence of bacterial resistance provided that the organisms are fully sensitive to both drugs. If, however, the organisms are resistant to one of the drugs, resistance will develop to the second as quickly as if that drug were being given alone (Turnbull, Wallace, Stewart, and Crofton, 1953). Evidence obtained in this unit (to be published elsewhere) suggests that it is important to use a routine sensitivity test which will detect even low degrees of resistance. In Great Britain the generally accepted routine test for detecting streptomycin resistance in tubercle bacilli is that of serial dilution in Dubos and Davis liquid medium as recommended by the Medical Research Council (1953c). But in this unit we have had a number of patients excreting organisms which appeared to be sensitive by the routine test, although other factors in the cases suggested that streptomycin-resistant bacilli had emerged. In the present report an alternative method is described for the detection of streptomycin resistance in *Myco. tuberculosis* which appears to be more sensitive than the previous routine test.

Material

All strains of *Myco. tuberculosis* used were obtained from the sputa of patients with pulmonary tuberculosis. The sputa were homogenized by the alkali-acid or tri-basic sodium phosphate method, and cultured on Löwenstein-Jensen medium. The time between the inoculation of the culture and the testing for streptomycin resistance varied from four weeks to five months.

The cultures used are grouped as follows:

(1) Twenty-nine cultures from 29 patients who had never received streptomycin, to show the normal variation of the method.

(2) Thirty-four cultures which on the routine test appeared to be sensitive to streptomycin although they came from 22 patients whose organisms were suspected of being resistant for one or more of the following reasons: (*a*) Streptomycin was known to have been given previously alone or in combination with other drugs to which the patients' organisms were known to be resistant. (*b*) Isoniazid (*iso*-nicotinic acid hydrazide) resistance had developed during combined treatment with streptomycin and isoniazid. All of these patients had previously received streptomycin alone or in combination with *p*-aminosalicylic acid (P.A.S.), when their organisms had already become resistant to P.A.S. (*c*) The occurrence of apparent sensitivity to streptomycin in spite of the fact that on previous occasions resistance to the drug had been found by the routine test.

(3) Fifteen cultures from 15 patients from whom previous cultures had been shown to be resistant to streptomycin by the routine test to determine whether any of these cultures might appear sensitive by the new test.

Methods

The streptomycin sensitivity of the cultures was tested by the routine liquid medium method and by a solid medium method simultaneously. In both cases the standard H37Rv strain of *Myco. tuberculosis* was used as the control organism. The results were reported as a resistance ratio, that is the ratio of the minimum inhibitory concentration for the test organism to the minimum inhibitory concentration for the control strain.

Routine Liquid Medium Test.—The routine method was that recommended by the Medical Research Council (1953c) using Dubos and Davis' "Tween 80"-albumin medium (Dubos and Davis, 1946). The drug concentrations used were 8, 4, 2, 1, 0.5, 0.25, and 0.125 μg. per ml. of medium. The tubes were inoculated from a second subculture of the organisms in Dubos and Davis medium. All tests were read after 10 days' incubation and some were also read after 28 days.

Solid Medium Test.—The organisms were tested for resistance to streptomycin on Löwenstein-Jensen medium at concentrations of 64, 32, 16, 8, 4, 2, 1, and 0.5 μg./ml. These concentrations represent the actual

* Royal Victoria Hospital Tuberculosis Trust Research Fellow in Bacteriology.

amount of streptomycin added to the medium. After the addition of the streptomycin approximately 2 ml. quantities were dispensed into ¼ oz. screw-capped bottles. These were inspissated in the horizontal position for one hour at 75 to 80° C. A suspension of a loopful of the growth of the primary culture of the organism was made in 0.3 ml. sterile distilled water by shaking with glass beads in a mechanical shaker. Using a nichrome wire loop of 3 mm. diameter, a loopful of the suspension was streaked up the centre of the slope. The slopes were incubated at 37° C. Readings were made at three, four, five, and six weeks. Growth was considered to be inhibited by the drug if fewer than 20 colonies appeared on the slope.

Solid Medium Test from the Liquid Medium Culture.—In order to ascertain whether resistance detected by the solid medium test but not by the liquid medium method was due to a failure of the resistant organisms to grow in the liquid medium subculture used for the routine test, a series of tests on solid medium was carried out using a loopful of the liquid medium subculture instead of the suspension. The number of organisms present in these two inocula is approximately the same.

Liquid Medium Tests without "Tween 80."—In order to ascertain whether the failure to detect resistance was due to the presence in the routine medium of "Tween 80," a series of tests was carried out in medium which did not contain any of this substance. The method used for these tests was exactly the same as for the routine test in all other respects. The liquid medium subculture from which the tests were inoculated contained "Tween 80."

Results

Cultures from Patients Who Had Never Received Streptomycin.—Table I shows the three and four weeks' readings of the solid medium tests on the 29 pre-treatment cultures. The results are expressed as resistance ratios. It will be seen that

TABLE I

NORMAL DISTRIBUTION OF RESISTANCE RATIOS ON SOLID MEDIUM TEST AFTER THREE AND FOUR WEEKS' INCUBATION

Incubation Period	No. of Cultures Showing Resistance Ratio						Total
	⅛	¼	½	1	2	4	
3 weeks ..	3	4	9	9	3	1	29
4 ,, ..	0	4	11	8	5	1	29

at three weeks the readings fell within the range ⅛ to 4. At four weeks this range was narrowed to ¼ to 4. Results at five and six weeks were similar to those found on the four weeks' reading. The range of inhibition for the test organisms varied from 1 to 16 μg./ml. and for the standard H37Rv strain from 4 to 8 μg./ml.

From the normal distributions the standard deviation of the three weeks' readings is 1.217 and of the four weeks' readings is 1.013. The limiting resistance ratio for P=0.01 is 8 for the three weeks' results and 4 at four weeks. In view of these results it was considered that a four-week reading would be the most satisfactory to use.

The 29 pre-treatment cultures were also tested by the liquid medium method. The results of both methods, expressed as resistance ratios, are compared in Table II. By each method 28 of the 29 cultures tested gave a resistance ratio of 2 or less. By each method one strain gave a resistance

TABLE II

COMPARISON OF RESULTS OF LIQUID AND SOLID MEDIA TESTS ON 29 CULTURES FROM 29 PATIENTS WHO HAD NEVER RECEIVED STREPTOMYCIN

Solid Medium Test	Liquid Medium Test				Total
Resistance Ratio	Resistance Ratio				
	½	1	2	4	
¼	3	1	0	0	4
½	5	6	0	0	11
1	0	6	1	1	8
2	0	5	0	0	5
4	0	1	0	0	1
Total ..	8	19	1	1	29

"Resistance ratio"=the ratio of the minimum inhibitory concentration for the test organism to the minimum inhibitory concentration for the standard H37Rv strain.

The standard strain was inhibited by concentrations varying from 0.25 to 0.5 μg/ml. in the liquid medium and from 4 to 8 μg./ml. in the solid medium.

The liquid medium tests were read at 10 days and the solid medium tests at four weeks.

ratio of 4. Both the patients from whom these latter two strains were isolated have subsequently been treated with streptomycin in combination with another drug. In neither case has their subsequent progress suggested that the organisms were resistant to streptomycin.

By the routine liquid medium test the minimum inhibitory concentration for the test organisms varied from 0.125 to 1.0 μg./ml. and that of the standard H37Rv strain from 0.25 to 0.5 μg./ml. From the normal distributions of the 29 cultures also tested by the solid medium method, the standard deviation is 0.591 and the limiting resistance ratio for P=0.01 is just over 2. Utilizing a further 41 cultures tested by the liquid method only, the limiting resistance ratio is again just over 2. Since the tests are carried out with twofold differences between the concentrations, this means that the lowest resistance ratio giving a 99 to 1 probability of resistance is 4.

Although the numbers in these series are rather small for statistical analysis, the results suggest that by either method a resistance ratio of 4 will

only be obtained with one pre-treatment culture in 100. Therefore a ratio of 4 by either method has been taken as indicative of the emergence of a resistant strain.

Cultures Appearing Sensitive by Routine Test although Suspected of Resistance.—Table III gives the results of the solid medium test on 34 cultures which were sensitive on the routine liquid medium test. In every patient from whom these cultures were obtained streptomycin resistance was suspected for the reasons shown. It will be seen that in only one culture was a sensitive result found on the solid medium test. Of the remaining 33 cultures, 10 gave a resistance ratio of 4, nine of 8, seven of 16, and seven of greater than 16.

TABLE III

RESULTS OF SOLID MEDIUM TESTS ON 34 CULTURES FROM 22 PATIENTS WITH SUSPECTED STREPTOMYCIN RESISTANCE ALTHOUGH SENSITIVE BY ROUTINE TEST

Reason for Suspecting Streptomycin Resistance	No. of Patients	No. of Cultures	Solid Medium Test — Resistance Ratio					
			1	2	4	8	16	>16
Previous unsatisfactory chemotherapy ..	9	13	0	0	5	1	5	2
Later resistance to second drug ..	3	5	1	0	2	1	0	1
Resistance previously shown by liquid medium test	10	16	0	0	3	7	2	4
Totals ..	22	34	1	0	10	9	7	7

Cultures Resistant by the Routine Test.—A comparison of the degree of streptomycin resistance found by the liquid and solid medium tests on cultures resistant by both methods is shown in Table IV. It will be seen that all the cultures showing resistance by the routine test also showed resistance when tested on the solid medium.

TABLE IV

COMPARISON OF DEGREE OF RESISTANCE BY LIQUID AND SOLID MEDIUM TESTS ON 15 CULTURES OF KNOWN RESISTANCE BY LIQUID MEDIUM TEST

Resistance Ratio in Solid Medium Test	Resistance Ratio in Liquid Medium Test				Total
	4	8	16	>16	
4	0	0	0	0	0
8	2	0	0	0	2
16	0	0	0	0	0
>16	3	2	2	6	13
Total	5	2	2	6	15

Cultures from Patients not Expected to Yield Streptomycin-resistant Organisms.—Twenty-four cultures, from patients in whom there was no reason to suspect the emergence of resistance, were all found to be sensitive by both methods.

Effect of Prolonged Incubation on Results of Liquid Medium Tests.—Table V shows the results of the liquid medium test after 10 and 28 days' incubation on 19 cultures which were resistant on the solid medium. It will be seen that in no case was the resistance ratio at 28 days more than twice that found at 10 days. No culture that gave a sensitive reading at 10 days appeared to be resistant at 28 days.

TABLE V

RESULTS OF LIQUID MEDIUM TEST AFTER 10 AND 28 DAYS' INCUBATION ON 19 KNOWN RESISTANT CULTURES

	28 Days' Reading Resistance Ratio	10 Days' Reading Resistance Ratio — Sensitive				Resistant				Total
		¼	½	1	2	4	8	16	>16	
Sensitive	¼	1	2	1	0	0	0	0	0	4
	1	0	1	1	3	0	0	0	0	5
	2	0	0	0	5	0	0	0	0	5
Resistant	4	0	0	0	0	0	0	0	0	0
	8	0	0	0	0	1	0	0	0	1
	16	0	0	0	0	0	0	0	0	0
	>16	0	0	0	0	0	0	0	4	4
	Total	1	3	2	8	1	0	0	4	19

Effect of "Tween 80" on Results of Liquid Medium Tests.—A comparison of the liquid medium tests carried out with and without "Tween 80" on 27 cultures that were resistant to streptomycin on the solid medium is shown in Table VI.

TABLE VI

RESULTS OF TESTS IN LIQUID MEDIUM WITH AND WITHOUT "TWEEN 80" ON 27 KNOWN RESISTANT CULTURES BY SOLID MEDIUM TEST

	Resistance Ratio in Liquid Medium Without "Tween 80"	Resistance Ratio in Liquid Medium with "Tween 80" — Sensitive		Resistant				Total
		1	2	4	8	16	>16	
Sensitive	1	0	0	0	0	0	0	0
	2	2	0	0	0	0	0	2
Resistant	4	2	7	1	0	0	0	10
	8	1	1	2	0	0	0	4
	16	0	1	0	0	0	0	1
	>16	0	1	3	1	1	4	10
	Total ..	5	10	6	1	1	4	27

Assuming that a resistance ratio of 4 indicates resistance by either method, it will be seen that of the 27 cultures tested two appeared to be sensitive when "Tween 80" was absent from the medium and 15 when "Tween 80" was present.

Comparison of Results of Solid Medium Test Inoculated from a Suspension of Primary Culture or Liquid Medium Subculture.—Table VII gives a comparison of the degrees of resistance found on solid medium tests inoculated from a suspension of the primary culture or from the subculture in the liquid medium. It will be seen that 37 of the 38 cultures were resistant by both methods. From only one culture was a sensitive result obtained from the liquid medium subculture, where a resistant result was found on the direct test.

TABLE VII

RESULTS OF SOLID MEDIUM TESTS ON 38 CULTURES ON A SUSPENSION OF PRIMARY CULTURE AND LIQUID MEDIUM SUBCULTURE

Resistance Ratio in Direct Test	Resistance Ratio in Test from Liquid Medium Subculture						Total
	1	2	4	8	16	>16	
4	1	0	2	3	1	1	8
8	0	0	3	2	2	1	8
16	0	0	2	2	2	0	6
>16	0	0	0	1	3	12	16
Total ..	1	0	7	8	8	14	38

Incidence of Resistance to P.A.S. and Isoniazid.—Of the 33 strains resistant to streptomycin on the solid medium test but not by the liquid medium method, 25 were resistant to P.A.S. and 22 to isoniazid.

Discussion

It is now well known that to use streptomycin in combination with isoniazid or P.A.S. in the treatment of tuberculosis only reduces the incidence of bacterial resistance to the second drug if the organisms are fully sensitive to streptomycin. It was therefore a little disturbing to find that in a number of cases the organisms appeared sensitive by the accepted routine liquid medium test, although other factors suggested that the organisms had developed streptomycin resistance. The value of streptomycin therapy in these cases was doubtful. A solid medium test was therefore tried in order to determine whether or not these cultures were in fact resistant to streptomycin. The results of these investigations are reported in this communication.

From the normal distribution curve of 29 cultures from patients who had never received streptomycin, it appears that in only one case in a hundred would a resistance ratio of 4 be found by the solid medium method. It therefore seems reasonable that for clinical practice a reading of 4 or above should be taken as indicating the development of resistance.

It was suggested by the Medical Research Council (1953c) that by the liquid medium test a resistance ratio of 8 was the lowest indicating resistance. The results of the present series suggest that a ratio of 4 is indicative of resistance 99 times out of 100. In fact the limiting resistance ratio is only just above 2 and if a narrower bracketing were used for the streptomycin concentrations a lower level might be found to be significant.

Tests on both solid and liquid medium were made on 34 cultures from patients in whom the existence of resistant tubercle bacilli was suspected either because of previous unsatisfactory chemotherapy, or because of resistance having been known to have been present on a previous occasion, or because resistance developed to a second drug during treatment with that drug and streptomycin in doses known to prevent the emergence of drug-resistant bacilli. All these cultures were sensitive by the liquid medium test. On solid medium 33 gave a reading of 4 or above, 23 of those being 8 or over. On only one culture from a case where there was reason to suspect the presence of streptomycin resistance was a sensitive result obtained on solid medium. Therefore in cases where resistance was shown by the solid medium method there was other evidence that resistance had developed. These results are in agreement with those reported by Holt and Cruickshank (1949) although they inoculated the solid medium tubes direct from the sputum concentrate.

All of the 15 cultures which showed resistance by the liquid medium test also appeared resistant by the solid medium test. It would therefore seem unlikely that by using the solid medium method a culture would be reported as sensitive which would have been resistant by the routine method.

The solid medium was not used as a screening method, and therefore there was less chance of detecting cultures resistant on the liquid medium but sensitive on the solid medium test. But of 24 cultures from patients in whom there was no reason to suspect the emergence of streptomycin resistance all were sensitive to the drug by both methods.

The question arises as to why there is this difference in the results of the two methods. It has been suggested that the detection of resistance by the solid medium test is due to the longer incubation period which allows of the appearance of the organisms with a slower growth rate. Results of the prolonged incubation of the liquid medium test do not support this theory. Of the 19

cultures tested, no culture giving a sensitive reading at 10 days appeared resistant after 28 days.

It has previously been found that the presence of "Tween 80" in the liquid medium enhances the inhibitory activity of streptomycin. Fisher (1948a) showed that of 20 strains tested in two types of liquid medium, 25% were resistant in Dubos and Davis' medium containing "Tween 80" whereas 55% were resistant when tested in Youmans medium where no "Tween" was used. This was also shown by testing strains for streptomycin sensitivity in a basal medium with the addition of "Tween 80" and of albumin singly and together (Fisher, 1948b). It was suggested that the use of 0.3% albumin in the Dubos and Davis' medium was sufficient to inactivate the "Tween 80" (Medical Research Council, 1948). In the present series only two of the 27 cultures resistant on solid medium were sensitive in the absence of "Tween 80," while 15 were sensitive when "Tween" was present, in spite of the addition of 0.3% of bovine albumin to the medium. Further work on this is being carried out.

It was possible that the differences in the results obtained in the liquid and solid media were due to a failure of the resistant organisms to grow in the liquid medium subculture from which the routine tests were inoculated. But resistance was demonstrated in 37 out of 38 cases where solid medium tests were inoculated from these subcultures.

The main disadvantage in using the Löwenstein-Jensen medium is the alleged breakdown of the streptomycin during inspissation. This might result in sensitive strains appearing resistant if a fixed minimum inhibitory concentration is used as indication of resistance. But the problem is largely overcome if the results are expressed as resistance ratios.

Of the 33 strains resistant to streptomycin on the solid medium but sensitive when tested in liquid medium, 25 were resistant to P.A.S. and 22 to isoniazid. The differences between the results of the streptomycin sensitivity tests by the two methods do not therefore seem to be related to resistance to either of these drugs.

In conclusion, it would seem that the solid medium test described will detect streptomycin resistance in tubercle bacilli more readily than will the routine liquid medium test.

Summary

A method is described for testing the streptomycin sensitivity of tubercle bacilli in Löwenstein-Jensen medium. The results are compared with those obtained in Dubos and Davis' liquid medium.

It is concluded that a resistance ratio of 4 by either method indicates resistance. Using this criterion, 33 of the 34 cultures tested from patients whose organisms were suspected of being resistant, were resistant by the solid medium test, although none were resistant by the liquid medium test.

There was no evidence that the solid medium test failed to detect resistance in cultures resistant by the liquid medium test.

The differences in the results of the two methods were not accounted for by the prolonged incubation of the solid medium test nor by the failure of the resistant organisms to grow in the liquid medium subculture.

The failure to demonstrate streptomycin resistance in the liquid medium may be due to the presence of "Tween 80."

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